Document made available under the Patent Cooperation Treaty (PCT)

International application number: PCT/IL05/000089

International filing date: 25 January 2005 (25.01.2005)

Document type: Certified copy of priority document

Document details: Country/Office: IL
Number: 160033
Filing date: 25 January 2004 (25.01.2004)

Date of receipt at the International Bureau: 21 February 2005 (21.02.2005)

Remark: Priority document submitted or transmitted to the International Bureau in compliance with Rule 17.1(a) or (b)

World Intellectual Property Organization (WIPO) - Geneva, Switzerland
Organisation Mondiale de la Propriété Intellectuelle (OMPI) - Genève, Suisse

PCT/IL 05 / 000089

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מספר: Number	160033
תאריך: Date	25 -01- 2004
הוקדם/נדחה Ante/Post-dated	

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(בעברית)
(Hebrew)
מערכת החדרה דרך העור של פולינוקליאוטידים

(באנגלית)
(English)
TRANSDERMAL DELIVERY SYSTEM FOR POLYNUCLEOTIDES

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מיום/dated	מיום/dated			

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TRANSDERMAL DELIVERY SYSTEM FOR POLYNUCLEOTIDES

מערכת החדרה דרך העור של פולינוקליאוטידים

TPH/006 IL

TRANSDERMAL DELIVERY SYSTEM FOR POLYNUCLEOTIDES

FIELD OF THE INVENTION

The present invention relates to a system for transdermal delivery of oligonucleotides or polynucleotides and to methods of use thereof. More specifically, the present invention relates to a system comprising an apparatus that generates micro-channels in the skin in conjunction with a pharmaceutical composition comprising an oligonucleotide or polynucleotide, wherein the oligonucleotide or polynucleotide can be delivered into the organism through the micro-channels. The system and methods of the present invention achieve expression of target proteins encoded by the polynucleotides, and hence are very useful in gene therapy.

BACKGROUND OF THE INVENTION

The direct introduction of a biologically active polypeptide into the cells of a patient can have significant therapeutic value. However, this approach also has several drawbacks. Of primary concern is the risk of potential toxicities, particularly at dosages sufficient to produce a biological response to the polypeptide. The clinical impact of a polypeptide is also limited by its relatively short half-life in vivo, which usually results from its degradation by proteases present in the target tissue. Moreover, polypeptides, which are injected into a tissue typically enter the blood circulation before they have a significant therapeutic effect on the tissue into which they were injected.

For these reasons, gene therapy is envisioned as a potentially definitive treatment for a variety of diseases or clinical conditions including cancer, genetic disorders, immune diseases, cardiovascular diseases, viral infections, and in clinical transplantation.

Clinical trials aiming at restoring defective genes have been currently performed for treating cancer. Thus, in lung cancer and head and neck cancer, for example, clinical trials have consistently showed evidence of p53 gene transduction and expression, apoptosis, and pathological complete responses (Moon et al. (2003) Clin. Cancer Res. 9: 5055-5067). Similarly, patients with severe combined immunodeficiency (SCID) treated with adenosine deaminase gene have shown significant immune reconstitution leading to protective immunity (Engel et al. (2003) Curr. Opin. Mol. Ther. 5: 503-507).

Additionally, silencing of undesired genes using antisense oligonucleotides directed against these genes also offers much hope for the treatment of a variety of diseases. For example, over expression of many growth factors was found to be correlated with cancer development. Antisense oligonucleotides against such growth factors have been shown to be useful in ameliorating cancer growth (Hirai et al. (2003) J. Gene Med. 5: 951-957).

It is clear, however, that gene therapy can be improved further. Promising avenues include improved gene delivery systems, design of immunogene and antiangiogenesis gene therapies, design of interfering RNA, and adjuvant use of gene therapy.

U.S. Pat. No. 5,749,847 to Zewert et al. discloses a method for delivering a nucleotide into an organism. The method includes applying a composition containing a nucleotide to epidermis of an organism, and then electroporating the epidermis so as to cause at least portion of the composition to pass across the epidermis and hence delivering the nucleotide to the organism.

U.S. Pat. No. 6,009,345 to Hofmann provides an apparatus and methods for transdermal delivery of drugs or genes that combine electroporation and iontophoresis. While electroporation forms new pathways through the stratum corneum, iontophoresis provides the driving force necessary to transport the drugs or genes through these pathways into the underlying tissue.

U.S. Pat. No. 6,527,716 to Eppstein discloses a method of delivering a nucleic acid into an organism, which includes ablating a biological membrane by the use of a heat conducting element and thereby porating the membrane in a selected area, applying an electromagnetic field to the selected area, and then contacting the selected area with a nucleic acid under conditions whereby the electromagnetic field actively induces the flux of the nucleic acid into the organism. Thus, ablating and forming micro pores in a biological membrane according to U.S. Pat. No. 6,527,716 involves a heat element that is held in contact with the biological membrane, and as the heat element absorbs energy it causes thermal ablation of the biological membrane, and the nucleic acid is delivered into the organism by the electromagnetic filed.

A need, therefore, exists for efficient apparatus and methods of transdermal delivery of nucleic acids, which do not require the provision of a driving force for the nucleic acids to be transported into the epidermis and dermis.

SUMMARY OF THE INVENTION

The present invention relates to an effective system for transdermal delivery of oligonucleotides or polynucleotides. The present invention further relates to a system and methods for ablating the stratum corneum of the skin and transdermally delivering oligonucleotides or polynucleotides to the pretreated skin. The system and methods of the present invention achieve expression of target proteins encoded by the polynucleotides, and hence are very useful in gene therapy.

It is now disclosed that the apparatus of the present invention generates micro-channels in the stratum corneum, the width of the micro-channels being in the range of several microns to tens of microns. This width enables large polynucleotides to be transported through the micro-channels and be delivered into the organism. The transport of the polynucleotides according to the invention does not require any driving force to enhance the flux of the polynucleotides. Rather, the micro-channels generated in the pretreated area of skin according to the present invention are of sufficient dimensions so as to provide enhanced flux of polynucleotides in the absence of electromagnetic field.

It is also disclosed that the transport of a polynucleotide through the micro-channels generated in the stratum corneum into the underlying tissues results in expression of a target protein encoded by the polynucleotide. It should be appreciated that the advantage of the apparatus of the invention resides in the formation of hydrophilic micro-channels, which permit transport of hydrophilic polynucleotides. Thus, the present invention does not require the use of liposomes to promote polynucloetide delivery and expression. According to the present invention, expression of a target protein is accomplished successfully with "naked" polynucleotides (not complexed to liposomes). Yet, the present invention is also useful with polynucleotides complexed with lipids.

According to one aspect, the present invention provides a transdermal delivery system comprising an apparatus that generates micro-channels in an area of the skin of a subject and a pharmaceutical composition comprising an oligonucleotide or polynucleotide. The oligonucleotide or polynucleotide are selected from oligonucleotides or polynucleotides of DNA, such as, for example, genomic DNA,

complementary DNA (cDNA), or synthetic DNA, and RNA such as mRNA or synthetic RNA, in a single stranded or double stranded form.

According to certain preferred embodiments, the oligonucleotide or polynucleotide is an oligonucleotide or polynucleotide that encodes at least one polypeptide, a protein, or a fragment thereof. Preferably, the oligonucleotide or polynucleotide encodes a therapeutic or immunogenic polypeptide selected from insulin, proinsulin, follicle stimulating hormone, insulin like growth factor-1, insulin like growth factor-2, platelet derived growth factor, epidermal growth factor, fibroblast growth factors, nerve growth factor, colony stimulating factors, transforming growth factors, tumor necrosis factor, calcitonin, parathyroid hormone, growth hormone, bone morphogenic protein, erythropoietin, hemopoietic growth factors, luteinizing hormone, calcitonin; glucagon; clotting factors, anti-clotting factors, atrial natriuretic factor, plasminogen activators, bombesin, thrombin, enkephalinase, vascular endothelial growth factor, interleukins, viral antigens, non-viral antigens, transport proteins, antibodies, and fragments thereof.

According to another preferred embodiment, the oligonucleotide or polynucleotide is operably linked to regulatory sequences, thereby the oligonucleotide or polynucleotide is capable of being expressed in cells of a treated subject.

According to other preferred embodiments, the oligonucleotide is an antisense oligonucleotide. Preferably, the antisense oligonucleotide comprises at least 15 nucleotides in length. More preferably, the antisense oligonucleotide comprises at least 21 nucleotides in length. It should be appreciated that the present invention also encompasses double stranded RNA (dsRNA) sequences, preferably small interfering RNA (siRNA) sequences. In a preferred embodiment, the siRNA sequence comprises at least 15 nucleotides in length. More preferably, the siRNA sequence comprises at least 21 nucleotides in length.

According to other preferred embodiments, the present invention comprises an apparatus, said apparatus comprises: (a) an electrode cartridge comprising at least one electrode; and (b) a main unit comprising a control unit which is adapted to apply electrical energy to the electrode when the electrode is in vicinity of the skin, typically generating current flow or one or more sparks, enabling ablation of stratum corneum in an area beneath the electrode, thereby generating at least one micro-channel. Preferably, the electrode cartridge comprises a plurality of electrodes capable of generating a plurality of micro-ehannels of uniform shape and dimensions. According to more

preferred embodiments, the electrical energy applied to the electrodes is of radio frequency.

According to another aspect, the present invention provides a method for transdermal delivery of an oligonucleotide or polynucleotide comprising: (a) generating at least one micro-channel in an area of the skin of a subject; and (b) introducing a pharmaceutical composition comprising a therapeutically effective amount of an oligonucleotide or polynucleotide. It should be appreciated that the method of the present invention is highly advantageous, for example, in replacement therapy in which expression of a therapeutic protein is required or in antisense therapy in which transcription and/or translation of a mutated or other detrimental protein should be arrested. It should be also appreciated that the method of the invention encompasses intradermal delivery of an oligonucleotide or polynucleotide, thus delivering locally the oligonucleotides or polynucleotides of the invention.

The present invention will be more fully understood from the following figures and detailed description of the preferred embodiments thereof.

BRIEF DESCRIPTION OF THE FIGURES

FIG. **1** shows scanning electron micrographs of RF-micro channels in intact human skin. Bar = 50 μm.

FIGS. **2A-B** show light micrographs of RF-micro channels in heat-separated epidermal membrane. A) Original magnification = X200; B) Original magnification = X100.

FIGS. **3A-F** show scanning electron micrographs of RF-micro channels in heat-separated epidermal membrane. FIG. **3A**, scanning electron micrograph at low magnification showing distribution pattern of channels following 2 applications of ViaDerm; FIGS. **3B-D**, scanning electron micrograph at high magnification showing dimensions of micro channels; FIGS. **3E-F** scanning electron visualization of micro channel depth using an angled electron beam (Bar (where added) = 50 μm).

FIG. **4** shows a calibration curve for calculation of donor concentrations of fluorescent nanoparticles. $R^2 = 0.990$.

FIGS. **5A-B** show transmission electron micrographs showing the validity for using fluorescent nanoparticles as a diffusive model for (lipid:polycation:DNA) LPD non-viral gene therapy vectors. FIG. **5A**, LPD 3:2:1 w/w vector FIG. **5B**, Fluorescent nanoparticle. Bar = 100 nm.

FIG. **6** shows the diffusion of fluorescent nanoparticles through ViaDerm-treated epidermal membranes. The data are presented as a percentage of total nanoparticles applied remaining in the donor phase after 16 hrs. Control, Non-treated epidermal membrane; 1, Epidermal membrane from fresh tissue treated with 2 applications of ViaDerm at parameter setting of 330V, 5 bursts, 700 μsec, 140 electrodes; 2, Epidermal membrane from fresh tissue treated with 2 applications of ViaDerm at parameter setting of 330V, 3 bursts, 700 μsec, 140 electrodes; 3, Epidermal membrane from defrosted tissue treated with 2 applications of ViaDerm at parameter setting of 330V, 5 bursts, 700 μsec, 140 electrodes; Filter, Filter paper only.

FIGS. 7**A-C** show stereomicrographs showing distribution pattern of RF-micro channels in human skin. FIG. 7A, Untreated skin; FIG. 7B-C, ViaDerm-treated skin. Bar = 500 μm.

FIGS. **8A-B** show light micrographs of methylene blue stained skin following ViaDerm treatment. FIG. 8A, Original magnification = X40; FIG. 8B, Original magnification = X100.

FIGS. **9A-C** show light micrographs of human breast skin treated with ViaDerm parameter setting of 330V, 3 bursts, 700 μsec, 140 electrodes. FIG. **9A**, Eosin stained, original magnification = X200; FIG. **9B**, Toludine blue stained, original magnification = X200; FIG. **9C**, Toludine blue stained, original magnification = X100. Bar = 100 μm.

FIGS. **10A-B** show light micrographs of human breast skin treated with ViaDerm parameter setting of 330V, 5 bursts, 700 μsec, 140 electrodes. FIG. **10A**, H&E stained, original magnification = X100; FIG. **10B**, H&E stained, original magnification = X200. Bar = 100 μm.

FIGS. **11A-D** show light micrographs of human breast skin treated with ViaDerm parameter setting of 290V, 1 burst, 700 μsec, 140 electrodes. FIG. **11A**, Eosin stained, original magnification = X100; FIG. **11B**, Eosin stained, original magnification = X100; FIG. **11C**, Toluidine blue stained, original magnification = X100; FIG. **11D**, Toludine blue stained, original magnification = X200. Bar = 100 μm.

FIGS. **12A-D** show light micrographs of human breast skin treated with ViaDerm parameter setting of 290V, 2 bursts, 700 μsec, 140 electrodes. FIG. **12A**, Eosin stained, original magnification = X100; FIG. **12B**, Eosin stained, original magnification = X100; FIG. **12C**, Toludine blue stained, original magnification = X100; FIG. **12D**, Toludine blue stained, original magnification = X100. Bar = 100 μm.

FIGS. **13A-D** show light micrographs of human breast skin treated with ViaDerm parameter setting of 290V, 5 bursts, 700 μsec, 140 electrodes. FIG. **13A**, Eosin stained, original magnification = X100; FIG. **13B**, Eosin stained, original magnification = X100; FIG. **13C**, Toludine blue stained, original magnification = X100; FIG. **13D**, Toludine blue stained, original magnification = X200. Bar = 100 μm.

FIGS. **14A-B** show light and fluorescent photomicrographs of RF-micro channels containing fluorescent nanoparticles. FIG. **14A**, a light photomicrograph; FIG. **14B**, an electron photomicrograph. Original magnification = X100. Bar = 100 μm.

FIGS. **15A-F** show fluorescent photomicrographs of RF-micro channels containing fluorescent nanoparticles. FIG.**15A**, Original magnification = X200; FIG. **15B-F**, Original magnification = X100. Bar = 100 μm.

FIGS. **16A-D** show fluorescent photomicrographs of ViaDerm-treated skin post-incubated with fluorescently labeled plasmid DNA alone. Original magnification = X200. Bar = 100 μm.

FIGS. **17A-F** show dual photomicrographs of ViaDerm-treated skin post-incubated with fluorescently labeled plasmid DNA in an LPD gene vector complex. FIGS. **17A**, **17C**, and **17E**, show light micrographs; FIGS. **17B**, **17D**, and **17F**, show fluorescence micrographs. Original magnification = X100. Bar = 100 μm.

FIGS. **18A-B** show light photomicrographs of heat-separated epidermal membrane stained for β-galactosidase expression. A) Original magnification = X100; B) Original magnification = X200.

FIG. **19** shows light photomicrographs of tape-stripped ViaDerm-treated human skin stained for β-galactosidase expression. Original magnification = X100.

FIGS. **20A-D** show light photomicrographs of H&E stained tissue sections from skin pre-treated with ViaDerm and assayed for β-galactosidase expression. FIG. **20A**, Original magnification = X100; FIG. **20B**, Original magnification = X200; FIG. **20C**, Original magnification = X100; FIG. **20D**, Original magnification = X200. Bar = 100 μm.

FIGS. **21A-B** show light photomicrographs of unstained tissue sections from skin pre-treated with ViaDerm and assayed for β-galactosidase expression. FIG. **21A**, Original magnification = X100; FIG. **21B**, Original magnification = X200. Bar = 100 μm.

DETAILED DESCRIPTION OF THE INVENTION

The present invention provides a transdermal delivery system for an oligonucleotide or polynucleotide comprising an apparatus that generates hydrophilic pathways within the stratum corneum in an area of the skin of a subject and a pharmaceutical composition, which comprises at least one oligonucleotide or polynucleotide. As oligonucleotides and polynucleotides are hydrophilic, the system of the invention is very useful in transdermal gene therapy.

Preparation of polynucleotides

As used herein "oligonucleotide" or "polynucleotide" refer to polymers of deoxyribonucleotides, ribonucleotides, and modified forms thereof in the form of a separate fragment or as a component of a larger construct, in a single strand or in a double strand form. The polynucleotides to be used in the invention include sense and antisense oligonucleotides or polynucleotides of DNA or RNA as appropriate to the goals of the therapy practiced according to the invention. The DNA or RNA molecules may be complementary DNA (cDNA), genomic DNA, synthesized DNA or a hybrid thereof or an RNA molecule such as mRNA. Accordingly, as used herein, the terms "DNA construct", "gene construct" "polynucleotide" and "oligonucleotide" are meant to refer to both DNA and RNA molecules. The term "oligonucleotide" refers to a polymer having not more than 50 nucleotides while the term "polynucleotide" refers to a polymer having more than 50 nucleotides. The terms oligonucleotides and polynucleotides are used in the invention interchangeably.

Oligonucleotides or polynucleotides for use in the invention can be obtained using hybridization methods well known in the art. DNA and RNA sequences may also be synthesized using automated nucleic acid synthesis equipment well known in the art. Use of the well-known polymerase chain reaction (PCR) is particularly preferred for generating mixtures of polynucleotides. Genomic nucleic acids may be prepared by

means well known in the art such as the protocols described in Ausubel, et al., Current Protocols in Molecular Biology, Chapters 2 and 4 (Wiley Interscience, 1989). cDNA can be synthesized according to means well known in the art (see, e.g., Maniatis, et al., Molecular Cloning; A Laboratory Manual (Cold Spring Harbor Lab, New York, 1982).

A polynucleotide can be deduced from the genetic code of a protein, however, the degeneracy of the code must be taken into account. Polyucleotides of the invention include sequences, which are degenerate as a result of the genetic code, which sequences may be readily determined by those of ordinary skill in the art.

Preferred polynucleotides for use in the present invention may operatively encode for therapeutic or immunogenic peptides or polypeptides. The immunogenic polypeptides can act as antigens to provoke a humoral and/or cellular response. The polynucleotides can also operatively encode for antibodies. In this regard, the term "antibody" encompasses whole immunoglobulin of any class, chimeric antibodies, hybrid antibodies with dual or multiple antigen specificities and fragments including hybrid fragments. Also included within the meaning of "antibody" are conjugates of such fragments, and so-called antigen binding proteins (single chain antibodies) as described, for example, in U.S. Pat. No. 4,704,692. Alternatively, the encoded antibodies can be anti-idiotypic antibodies (antibodies that bind other antibodies) as described, for example, in U.S. Pat. No. 4,699,880.

Therapeutic polypeptides according to the invention include, but are not limited to, insulin, proinsulin, follicle stimulating hormone, insulin like growth factor-1 and insulin like growth factor-2, platelet derived growth factor, epidermal growth factor, fibroblast growth factors, nerve growth factor, transforming growth factors, tumor necrosis factor, calcitonin, parathyroid hormone, growth hormone, bone morphogenic protein, erythropoietin, hemopoietic growth factors and luteinizing hormone, calcitonin; glucagon; clotting factors such as factor VIIIC, factor IX, tissue factor, and von Willebrand factor; anti-clotting factors such as Protein C; atrial natriuretic factor; lung surfactant; a plasminogen activator, such as urokinase or tissue-type plasminogen activator, including human tissue-type plasminogen activator (t-PA); bombesin; thrombin; enkephalinase; a collagen; a collagen domain; mullerian-inhibiting agent; relaxin A-chain; relaxin B-chain; prorelaxin; Dnase; inhibin; activin; vascular endothelial growth factor; receptors for hormones or growth factors; integrin; protein A or D; rheumatoid factors; a neurotrophic factor such as bone-derived neurotrophic factor (BDNF), neurotrophin-3, -4, -5, or -6 (NT-3, NT-4, NT-5, or NT-6, CD proteins such as

CD-3, CD-4, CD-8, and CD-19; osteoinductive factors; immunotoxins; an interferon such as interferon-alpha, -beta, and -gamma; colony stimulating factors (CSFs), e.g., M-CSF, GM-CSF, and G-CSF; interleukins (ILs), e.g., IL-1 to IL-10; superoxide dismutase; T-cell receptors; surface membrane proteins; decay accelerating factor; viral antigen such as, for example, a portion of the AIDS envelope; transport proteins; homing receptors; addressins; regulatory proteins; antibodies; and fragments thereof.

Those of skill in the art will, however, appreciate that the methods of the invention may be adapted for use in administering any oligonucleotide, polynucleotide, or mixture thereof, which operatively encode therapeutic and/or immunogenic peptides of interest. The invention is therefore not limited to use with any particular polynucleotide or oligonucleotide(s).

The oligonucleotides or polynucleotides of the invention may contain a modified internucleoside phosphate backbone to improve the bioavailability and hybridization properties of the oligonucleotide or polynucleotide. Linkages are selected from the group consisting of phosphodiester, phosphotriester, methylphosphonate, phosphoroselenoate, phosphorodiselenoate, phosphoroanilothioate, phosphoroanilidate, phosphoramidate, phosphorothioate, phosphorodithioate or combinations thereof.

Additional nuclease linkages include alkylphosphotriester such as methyl- and ethylphosphotriester, carbonate such as carboxymethyl ester, carbamate, morpholino carbamate, 3'-thioformacetal, silyl such as dialkyl (C_1 -C_6)- or diphenylsilyl, sulfamate ester, and the like. Such linkages and methods for introducing them into oligonucleotides are described in many references, e.g. reviewed generally by Peyman and Ulmann, Chemical Reviews, 90:1543-584 (1990).

As used herein, the term "gene construct" refers to a DNA or RNA molecule that comprises an oligonucleotide or polynucleotide which encodes a target protein and which includes initiation and termination signals operably linked to regulatory elements including a promoter and polyadenylation signal capable of directing expression in the cells of a subject. Thus, a gene construct contains the necessary regulatory elements operably linked to the polynucleotide that encodes a target protein, such that when present in a cell of the individual, the polynucleotide will be expressed.

The regulatory elements necessary for gene expression of a DNA molecule include: a promoter, an initiation codon, a stop codon, and a polyadenylation signal. In addition, enhancers are often required for gene expression. It is necessary that these elements be operably linked to the polynucleotide that encodes the target protein such

that the polynucleotide can be expressed in the cells of a subject and thus the target protein can be produced.

Initiation codons and stop codons are generally considered to be part of a gene construct comprising the polynucleotide that encodes the target protein. However, it is necessary that these elements are functional in the subject to which the polynucleotide is administered.

Examples of promoters useful to practice the present invention include, but are not limited to, promoters from Simian Virus 40 (SV40), Mouse Mammary Tumor Virus (MMTV) promoter, Human Immunodeficiency Virus (HIV) such as the HIV Long Terminal Repeat (LTR) promoter, Moloney virus, ALV, Cytomegalovirus (CMV) such as the CMV immediate early promoter, Epstein Barr Virus (EBV), Rous Sarcoma Virus (RSV), promoters from human genes such as human actin, human myosin, human hemoglobin, human muscle creatine and human metalothionein and tissue-specific promoters such as involucrin, keratin 5, and keratin 14. Suitable protocols for use of promoters in construction of gene constructs are well known in the art (see, for example, Current Protocols in Molecular Biology, Chapter 1 (Wiley Interscience, 1989)) and are exemplified herein below.

Examples of polyadenylation signals useful to practice the present invention include, but are not limited to, SV40 polyadenylation signals and LTR polyadenylation signals.

In addition to the regulatory elements required for DNA expression, other elements may also be included in the gene construct. Such additional elements include enhancers. The enhancer may be selected from the group including, but not limited to, human actin, human myosin, human hemoglobin, human muscle creatine and viral enhancers such as those from CMV, RSV and EBV.

Gene constructs can be provided with mammalian origin of replication in order to maintain the construct extrachromosomally and produce multiple copies of the construct in the cell. Plasmids pCEP4 and pREP4 from Invitrogen (San Diego, Calif.) contain the Epstein Barr virus origin of replication and nuclear antigen EBNA-1 coding region, which produces high copy episomal replication without integration. Other plasmids known in the art may be used so long as the gene constructs express the target protein encoded by the polynucleotide sequence.

In order to be a functional gene construct, the regulatory elements must be operably linked to the polynucleotide that encodes the target protein. Accordingly, it is

necessary for the initiation and termination codons to be in frame with the coding sequence.

By inserting one or more sequences of interest into a gene construct, along with another gene, which encodes a ligand for a receptor on a specific target cell, for example, the gene construct is now target specific. Gene constructs can be made target specific by inserting, for example, a polynucleotide encoding a sugar, a glycolipid, or a protein. Preferred targeting is accomplished by using an antibody to target the gene construct. Those of skill in the art will know of, or can readily ascertain without undue experimentation, specific polynucleotide sequences which can be inserted into the gene construct to allow target specific delivery of the gene construct containing the polynucleotides of interest.

Early work showed that polycations such as polylysine and DEAE-dextran promote the uptake of proteins and single- and double-stranded polynucleotides into animal cells. For example, polylysine-based vectors have been extensively tested for gene transfer. Thus, the present invention also encompasses synthetic DNA-delivery systems. In a currently preferred embodiment, the polycation is a protamine sulfate.

According to other preferred embodiments of the invention, introducing a polynucleotide to a subject' skin is accomplished by lipid complexing rather than by the "naked" polynucleotide sequence. Lipids for promoting DNA delivery are well known in the art and may be selected from the group consisting of phospholipids, e.g., phosphatidylethanolamines and phosphatidylcholines such as 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine, 1,2-diphytanoyl-sn-glycero-3-phosphoethanolamine, and 1,2-dimyristoyl-glycero-3-phosphoethanolamine. Alternatively, in a currently preferred embodiment the lipid is 1,2-Dioleoyl-3-trimethylammonium-propane. The present invention also comprises polynucleotide compositions wherein the composition comprises one or more lipids. According to the present invention, the lipids may be mixed or combined with a polynucleotide in a number of ways to produce a variety of compositions of non-covalently bonded macroscopic structures, e.g., liposomes, multilamellar vesicles, unilamellar vesicles, micelles, and simple films. The polynucleotide and lipids can be mixed in a variety of molar ratios. A protocol for complexation of polynucleotide, lipid, and polycation is exemplified herein below (see Example 7). However, other procedures as known in the art may be useful so long as the polynucleotide is capable of being expressed within cells of a treated subject.

It should be appreciated that the introduction of a gene construct comprising a polynucleotide encoding a target protein that is operably linked to regulatory sequences brings about the expression of the target protein in a treated subject. This may be used in replacement therapy in which a gene encoding a target protein is introduced into target cells of a subject, thus resulting in the production of the protein necessary to forestall development of a disorder associated with a deficiency of this protein. This may also be used in replacement therapy in which a mutant gene is expressed within target cells of a subject and a wild type gene is introduced into the target cells, thus resulting in the production of the wild type protein necessary to forestall development of the disorder associated with the mutant protein.

Another particular advantage of the invention will be its use in antisense therapy. Briefly, where a particular disorder is associated with the expression of a particular mutated DNA sequence, a polynucleotide sequence that interferes with the specific expression of the mutated gene at the transcriptional or translational level can be used. This approach utilizes, for example, antisense nucleic acid and/or ribozymes to block transcription or translation of a specific mutated mRNA, either by masking that mRNA with an antisense nucleic acid or by cleaving it with a ribozyme.

Thus, antisense nucleic acids are DNA or RNA molecules that are complementary to at least a portion of a specific mRNA molecule (Weintraub, Scientific American, 262:40, 1990). As such, in the cell, the antisense nucleic acids hybridize to the corresponding mRNA, thus forming a double-stranded molecule. The antisense nucleic acids interfere with the translation of the mRNA, since the cell will not translate an mRNA that is double-stranded. To date, several genes and oncogenes have been targeted for suppression or down-regulation by antisense nucleic acids including, but not limited to, p53, ras, fos, and myc.

Ribozymes are RNA molecules possessing the ability to specifically cleave other single-stranded RNA in a manner analogous to DNA restriction endonucleases. Through the modification of polynucleotide sequences that encode these RNAs, it is possible to engineer molecules that recognize specific polynucleotide sequences associated with production of a mutated proto oncogene or tumor suppressor gene in an RNA molecule and cleave it (Cech, J. Amer. Med. Assn., 260:3030, 1988). A major advantage of this approach is that, because ribozymes are sequence-specific, only target mRNAs with particular mutant sequences are inactivated.

The present invention, therefore, encompasses double stranded RNA (dsRNA), and more particularly small interfering RNA (siRNA), which are known to mediate sequence specific mRNA degradation. As 21-nucleotide siRNA duplexes were found to be highly selective and sequence-specific inhibitors of endogenous genes, the present invention encompasses siRNA sequences comprising at least 15 nucleotides, more preferably at least 21 nucleotides.

Pharmaceutical compositions

The pharmaceutical compositions of the invention comprise a therapeutically effective amount of at least one oligonucleotide or polynucleotide of the invention.

As the pharmaceutical composition is hydrophilic, the pharmaceutical composition may also include diluents of different buffer content (e.g., Tris-HCl, phosphate, citrate), pH and ionic strength. The pharmaceutical composition may also include additives such as albumin to prevent adsorption to surfaces, anti-oxidants (e.g., ascorbic acid, sodium metabisulfite), stabilizers (e.g. monosaccharides and disaccharides such as glucose, galactose, and sucrose) and preservatives (e.g., Thimerosal, benzyl alcohol, parabens, m-cresol). Administration of pharmaceutically acceptable salts of the polynucleotides described herein is also encompassed in the present invention. Salts derived from inorganic bases include sodium, potassium, lithium, ammonium, calcium, magnesium, and the like. Salts derived from pharmaceutically acceptable organic non-toxic bases include salts of primary, secondary, and tertiary amines, basic amino acids, and the like.

The pharmaceutical composition may also include hydrophilic polymers. The hydrophilic polymers may increase the half-lives of the polynucleotides of the invention and hence increase their bioavailability. The hydrophilic polymer is selected from the group consisting of cellulose, hydroxy cellulose, carboxymethyl cellulose, hydroxyethyl cellulose, hydroxypropyl cellulose, hydroxypropylmethyl cellulose, polysaccharides, chitin, chitosan, diacylated chitin, gum acacia, agarose, carrageenan, gelatin, gum tragacanth, alginate, karaya gum, veegum, pectin, hyaluronic acid, maltodextrin, polyvinylpyrrolidone, polyglycolic acid, polyoxyethylene, polyoxypropylene, polyacrylates, methacrylate polymers, and a like.

The pharmaceutical composition may further include at least one nuclease inhibitor to prevent the polynucleotide of the invention from being degraded by nucleases, e.g. aurintricarboxylic acid (ATA).

The pharmaceutical composition may also include at least one protein or polypeptide. For example, the large T-antigen nuclear localization signal is a protein that binds DNA and facilitates its transport into the nucleus of the cell. Thus, the combination of a polynucleotide according to the invention and a protein known to enhance DNA transport is encompassed in the present invention.

The formulation of the pharmaceutical composition comprising a therapeutically effective amount of an oligonucleotide or polynucleotide according to the present invention is determined so as to provide improved stability of the oligonucleotide or polynucleotide while retaining or improving its bioavailability.

The pharmaceutical composition may be formulated as an aqueous solution. Alternatively, the pharmaceutical composition may be in a dry or lyophilized formulation as disclosed in PCT/IL03/00903, which is incorporated herein by reference as if fully set forth. The composition may also be formulated in a medical patch as known in the art or as disclosed in PCT/IL03/00903 and in PCT/IL03/00902, the content of which is incorporated by reference as if fully set forth. The pharmaceutical composition may be formulated in a form of a film, or a gel using, for example, Carbopol® 940 and triethanolamine as gelling agents, or may the pharmaceutical composition may be formulated in any other suitable form, which enables transdermal delivery of the polynucleotide of the invention. .

The dosage of each polynucleotide to be administered to a subject using the method of the invention will vary depending on the desired response and the polynucleotide used.

The dosage of the polynucleotide may be modified to achieve therapeutic levels of expression. Means to confirm the presence of DNA or RNA and quantity of expressed proteins are well known to those skilled in the art. Certain such means are illustrated in the Examples provided herein below; generally, they include immunoassays (such as enzyme-linked immunosorbent assays), PCR techniques, and immunohistological analyses performed according to techniques, which are well known in the art. Dosages of the administered polynucleotides can be adjusted to achieve the desired level of expression based on information provided by these detection and quantification means as well as on in vivo clinical signs known to practitioners skilled in the clinical art.

Devices for enhancing transdermal delivery of polynucleotides

The system of the present invention further contains an apparatus for enhancing transdermal delivery of a polynucleotide. According to the principles of the invention the apparatus is used to generate a micro-channel through which a hydrophilic pharmaceutical composition is delivered efficiently.

In a preferred embodiment of the present invention, the apparatus for enhancing transdermal movement of a substance using radio frequency (RF) energy is as disclosed in U.S. Pat. No. 6,148,232; U.S. Pat. No. 6,597,46 and in U.S. No. 6,611,706 comprising: an electrode cartridge, optionally removable, comprising at least one electrode, and a main unit comprising a control unit. The main unit loaded with the electrode cartridge is also denoted herein ViaDerm.

The control unit is adapted to apply electrical energy to the electrode typically by generating current flow or one or more sparks when the electrode cartridge is in vicinity of the skin. The electrical energy in each electrode within the electrode array causes ablation of stratum corneum in an area beneath the electrode, thereby generating at least one micro-channel.

The term "micro-channel" as used in the context of the present patent application refers to a pathway, generally extending from the surface of the skin through all or significant part of the stratum corneum, through which molecules can diffuse.

The control unit comprises circuitry which enables to control the magnitude, frequency, and/or duration of the electrical energy delivered to an electrode, in order to control current flow or spark generation, and consequently to control the dimensions and shape of the resulting micro-channel. Typically, the electrode cartridge is discarded after one use, and as such is designed for easy attachment to the main unit and subsequent detachment from the unit.

To minimize the chance of contamination of the cartridge and its associated electrodes, attachment and detachment of the cartridge is performed without the user physically touching the cartridge. Preferably, cartridges are sealed in a sterile cartridge holder, which is opened immediately prior to use, whereupon the main unit is brought in contact with a top surface of the cartridge, so as to engage a mechanism that locks the cartridge to the main unit. A simple means of unlocking and ejecting the cartridge, which does not require the user to touch the cartridge, is also provided.

For still other applications, an integrated electrode/medicated pad cartridge is used, to provide a practical apparatus as disclosed in International Patent Application

No. WO 02/092163, which is assigned to the assignee of the present patent application and incorporated herein by reference, is also denoted MicroDerm. In these applications, the cartridge comprises an electrode array, a controlled unit and a medicated pad. Accordingly, no template is typically required. The user places the electrodes against the skin and this contact is sufficient to initiate current flow or spark formation within the electrode and the subsequent formation of micro-channels. An adhesive strip, coupled to the bottom of the medicated pad, comes in contact with and sticks to the skin when the electrodes are placed against the skin. A top cover on the medicated matrix is coupled to the electrode region of the cartridge, such that as the electrode region, fixed to the handle, is removed from the skin the top cover is pulled off the medicated pad and the pad is concurrently folded over the treated region of skin. This type of application eliminates the need for the user to touch any parts of the electrode cartridge or the medicated pad, thus substantially reducing or eliminating the likelihood of the user contaminating the apparatus.

In a preferred embodiment, current may be applied to the skin in order to ablate the stratum corneum. In one preferred embodiment, spark generation, cessation of spark generation, or a specific current level may be used as a form of feedback, which indicates that the desired depth has been reached and current application should be terminated. For these applications, the electrodes are preferably shaped and/or supported in a cartridge that is conducive to facilitating ablation of the stratum corneum and the epidermis to the desired depth, but not beyond that depth. Alternatively, the current may be configured so as to ablate the stratum corneum without the generation of sparks.

Generally preferred embodiments of the present invention typically incorporate methods and apparatus described in U.S. Pat. No. 6,611,706 entitled "Monopolar and bipolar current application for transdermal drug delivery and analyte extraction". For example, this application describes maintaining the ablating electrodes either in contact with the skin, or up to a distance of about 500 microns therefrom. The application further describes spark-induced ablation of the stratum corneum by applying a field having a frequency between about 10 kHz and 4000 kHz, preferably between about 10 kHz and 500 kHz.

In some preferred embodiments of the present invention, the cartridge supports an array of electrodes, preferably closely spaced electrodes, which act together to produce a high micro-channel density in an area of the skin under the cartridge. Typically,

however, the overall area of micro-channels generated in the stratum corneum is small compared to the total area covered by the electrode array.

Methods for transdermal delivery of polynucleotides

The present invention provides a method for transdermal delivery of an oligonucleotide or polynucleotide comprising generating at least one micro-channel in an area of the skin of a subject, and introducing to the area of skin in which the micro-channels are present a pharmaceutical composition comprising a therapeutically effective amount of an oligonucleotide or polynucleotide.

The term "therapeutically effective amount" means the amount of a polynucleotide sufficient to produce the desired effect when applied topically over the duration of intended use.

According to preferred embodiments of the invention, the method of transdermal delivery of a polynucleotide is very useful for replacement therapy, whereby a target protein encoded by the polynucleotide is expressed within cells of a subject thereby producing the target protein.

According to additional preferred embodiments of the invention, the method of transdermal delivery of a polynucleotide is highly useful in antisense therapy wherein a mutant gene is expressed within target cells and the expression of the mutant gene causes a disease. Thus, introducing an antisense oligonucleotide abrogates the expression of the mutant gene.

It should be appreciated that the method of the invention also encompasses intradermal delivery of polynucleotides, thus delivering the polynucleotides of the invention locally.

EXAMPLE 1

Morphology of RF-micro channels in full-thickness human skin

Materials and Methods

Tissue:

Full-thickness human breast skin was obtained from mastectomy or breast reduction with local ethical committee approval and informed patient consent. All excess adipose tissue was removed by blunt dissection. Tissue was transported in

MEM (EAGLES) 25MM HEPES growth media (Cat. No. 32360-026, Invitrogen Corporation, Paisley, UK) and used within 3 hrs of excision.

ViaDerm parameters:

ViaDerm parameter setting: 2 applications; Electrodes: 140; Burst length: 700μsec; Number of bursts: 5; TEWL control: 8.8 g/hm^2; TEWL after application(s): 11.4 g/hm^2

Electron microscopy:

The samples were fixed with 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 60 min at room temperature and washed for 10 min (2x5 min) in the same buffer. The samples were post-fixed in 1% osmium tetroxide in 0.1 M cacodylate buffer for 1 hr at 4°C and then dehydrated with a graded series of ethanol concentrations as follows: 70% for 10 min at 4°C; 100% for 10 min at 4°C; 100% for 10 min at 4°C; 100% for 10 min at 4°C. The samples were then transferred into a critical point drier (Samdri 780, Maryland, USA) and dried using carbon dioxide for 12 hrs. The samples were mounted on metal stubs and coated with a thin layer of pure gold using an Edward sputter coater prior to examination in a Philips XL-20 scanning electron microscope.

Results

FIG. **1** shows the structural morphology of the channels created in full-thickness breast skin following application of ViaDerm. The channels clearly appear as deep invaginations into the tissue with the width of the channel ranging from 30 to 50μm.

EXAMPLE 2

Morphology of micro channels in heat-separated epidermal membrane

The distribution pattern and dimensions of RF-micro channels in heat-separated human epidermal membrane.

Materials and Methods

Tissue:

Full-thickness human skin was obtained and prepared as described in Example 1.

ViaDerm parameters:

ViaDerm parameter setting: 2 applications; Electrodes: 140; Burst length: 700μsec; Number of bursts: 5; TEWL control: 6.2 g/hm^2; TEWL after application(s): 20.6 g/hm^2

Heat separation of skin:

Following application of the ViaDerm device the upper layers (stratum corneum and viable epidermis) of the skin were removed using the following technique: a glass container of distilled water was warmed to 60°C and the skin sample was cut to an appropriate size for heat separation. The tissue was placed into the pre-heated container using forceps and after 55 secs, the tissue was removed using forceps and allowed to dry. A small incision through the stratum corneum and epidermal layer was made using a scalpel. The dermis of the tissue was then gripped. At the point of incision, the epidermal and stratum corneum membrane were slowly peeled away from dermal layer. Once the membrane 'flap' was large enough, the remaining area of the membrane was removed using a gloved hand. The isolated membrane was placed in a container of distilled water at room temperature, where the membrane unfolded and floated on the surface of the water, with the stratum corneum facing upward. A piece of foil was placed underneath the membrane and the membrane was removed from the bath in its flattened form.

Light microscopy:

The epidermal membrane was placed onto a glass microscope slide and viewed using an Olympus IX50 microscope under brightfield illumination.

Electron microscopy:

Dried heat-separated membrane was mounted on a metal stub and coated with a thin layer of pure gold using an Edward sputter coater prior to examination in a Philips XL-20 scanning electron microscope.

Results

FIG. 2 and FIG. 3 show that the RF-micro channels either totally penetrate or partially penetrate the heat-separated epidermal membrane comprising of stratum corneum and viable epidermis. Although the depth of the micro channels was variable, probably due to variation in thickness of the separated membrane, the diameter of the micro channels, approximately 50 μm, was reproducible and consistent with the micro channel dimensions observed in full-thickness skin (FIG. 1).

EXAMPLE 3

Diffusion of fluorescent nanoparticles through micro channels in heat-separated epidermal membrane

The diffusion of fluorescent nanoparticles through ViaDerm-treated heat-separated epidermal membrane was performed. These fluorescent nanoparticles have similar particle diameter to non-viral gene therapy vectors. In addition, it was aimed to determine whether the diffusion characteristics depend on the parameter setting employed and whether the skin has to be analyzed fresh or following frozen storage.

Materials and Methods

Tissue:

Fresh full-thickness human skin was obtained and prepared as described in Example 2. In addition, a skin sample that had been obtained and transported in the same way prior to freezing at -20°C for 6 weeks was defrosted at room temperature.

ViaDerm parameters:

Sample 1: (fresh tissue) ViaDerm parameter setting: 2 applications; Electrodes: 140; Burst length: 700µsec; Number of bursts: 5; TEWL control: 6.7 g/hm^2; TEWL after application(s): 28.2 g/hm^2.

Sample 2: (fresh tissue) ViaDerm parameter setting: 2 applications; Electrodes: 140; Burst length: 700µsec: Number of bursts: 3; TEWL control: 6.2 g/hm^2; TEWL after application(s): 19.2 g/hm^2.

Sample 3: (frozen tissue) ViaDcrm parameter setting: 2 applications; Electrodes: 140; Burst length: 700µsec; Number of bursts: 5; TEWL control: 6.5 g/hm^2; TEWL after application(s): 9.3 g/hm^2.

Heat separation:

Heat-separated epidermal membrane was prepared as described in Example 2.

Diffusion assay:

Non-treated and ViaDerm-treated human epidermal membranes were placed on Type-1 filter paper (Whatman PLC, Maidstone, Kent) and mounted between donor and receptor compartments of static Franz-type glass diffusion cells. The compartments were sealed using silicone grease, and metal clips used to clamp the chambers tightly closed. The receptor phase of each cell, which had a precisely calibrated volume and diffusional area, was filled with Phosphate Buffered Saline (PBS; pH 7.4; Sigma

Chemical Company, Poole, UK) and a magnetic follower was added. The receptor arm was sealed with a glass cap and the donor chamber was occluded with a cover slip to prevent sample evaporation. The cells were placed on a multipoint magnetic stirrer in a water-bath maintained at a constant temperature of 37°C, to provide continuous agitation and a skin surface temperature of 32°C. Prior to addition of the test formulations to the donor chamber, cells were allowed to equilibrate for at least 30 minutes and the integrity of epidermal membranes was visually inspected.

Fluorescently (FITC) labeled polystyrene nanospheres (L-1280; Sigma Chemical Company, Poole, UK) were added to the donor chambers of diffusion cells. In three of the cells the epidermal membranes had been pre-treated with 2 applications of ViaDerm. A positive control cell was prepared using filter paper alone. A negative control was prepared using a diffusion cell containing an untreated epidermal membrane.

A volume of 500 µl of a 10 µl/ml dilution of the fluorescent nanospheres stock suspension, concentration 4.57^{10}/µl, was applied to the surface of the epidermal membrane. After 16 hrs the donor phase was transferred to a 96 well plate for analysis using a fluorescence spectrophotometer (BMG Fluostar, Aylesbury, UK) with excitation and emission wavelengths set at 485 and 520 nm respectively. A calibration curve was performed using standard dilutions of the suspension of fluorescent nanoparticles (**FIG. 4**).

Transmission electron microscopy (TEM):

15µl of a suspension of lipid:polycation:pDNA (LPD) complexes or fluorescent nanoparticles was placed onto 100 mesh nickel grids. After 3 min the excess solution was wicked off with filter paper and replaced with freshly filtered and centrifuged 2% aqueous uranyl acetate for 30 secs. The grids were washed twice with distilled water, allowed to dry and imaged using a Philips 208 transmission electron microscope.

Results

FIG. **5** illustrates the rationale for using spherical fluorescent nanoparticles as a diffusive model for LPD non-viral gene delivery vectors. FIG. **6** shows the diffusion of fluorescent nanoparticles through ViaDerm-treated epidermal membranes. As shown in FIG. **6**, the untreated epidermal membrane sample (control) demonstrated a highly significant barrier function to 100 nm nanoparticles, with 94.3% of the applied nanoparticles still being recoverable in the donor phase after 16 hrs incubation. All of

the ViaDerm-treated epidermal membranes demonstrated an increased and reproducible permeability to the nanoparticles, with between 28.5 and 30.4% of the applied nanoparticles leaving the donor compartment via the RF-micro channels. This increase in diffusion of nanoparticles is considerable especially when compared with the positive control (filter). In the filter sample, 44.8% of the fluorescent nanoparticles were still present in the donor compartment reflecting the barrier properties of the underlying filter support. The transepidermal water loss (TEWL) data for the skin samples was intriguing. Despite the diffusion data showing comparable levels of permeation in the outer layers of the skin for both fresh and frozen tissue, the fresh skin experienced an increase in TEWL from 6.7 to 28.2 g/hm^2 when treated with the ViaDerm whereas previously frozen tissue showed a smaller increase from 6.5 to 9.3 g/hm^2 at the same parameter setting. This reduced increase in TEWL following application of ViaDerm was observed for other frozen samples and it was therefore decided to use freshly obtained unfrozen tissue for all subsequent experiments.

EXAMPLE 4

Visualization of micro channels *en face*

The distribution pattern of RF-micro channels in full-thickness human skin was detected.

Materials and Methods

Tissue:

Full-thickness human skin was obtained and prepared as described in Example 1.

ViaDerm parameters:

FIG. **7B**: ViaDerm parameter setting: 2 applications; Electrodes: 140; Burst length: 700μsec; Number of bursts: 5; TEWL control: 8.1 g/hm^2; TEWL after application(s): 18.0 g/hm^2.

FIG. **7C**: ViaDerm parameter setting: 2 applications; Electrodes: 140; Burst length: 700μsec; Number of bursts: 5; TEWL control: 9.5 g/hm^2; TEWL after application(s): 22.0 g/hm^2.

FIG. **8**: ViaDerm parameter setting: 1 application; Electrodes: 140; Burst length: 700μsec; Number of bursts: 5; TEWL control: 6.8 g/hm^2; TEWL after application(s): 7.6 g/hm^2.

Sample preparation:

Skin was incubated in media (MEM (EAGLES) 25MM HEPES) for 24 hrs at 37°C. Following two washes in PBS the skin was fixed in 0.5% gluataraldehyde for 2 hrs on ice. For methylene blue staining, 5 drops of 1.5% methylene blue solution was applied to the surface of the ViaDerm treated skin for 5 min. Subsequently, excess stain was rinsed with PBS and the tissue surface was swabbed with 70% ethanol.

Stereomicroscopy:

Tissue was visualized using a Zeiss Stemi 2000C Stereomicroscope with a 2,0X attachment and an Scholt KL1500 electronic light source.

Light microscopy:

Tissue stained with methylene blue was visualized using an Olympus BX50 microscope and an Scholt KL1500 electronic light source.

Results

FIG. **7B** and FIG. **7C** clearly demonstrate the distribution of micro channels in ViaDerm treated skin. The brown discoloration of the channels observed in FIG. 7 suggests a change in the tissue properties surrounding the channel. The channels can also be visualized through their ability to uptake and retain methylene blue in solution (FIG. **8**).

EXAMPLE 5

Histology of sectioned tissue

The depth and structural morphology of ViaDerm-treated human skin was next performed.

Materials and Methods

Tissue:

Full-thickness human skin was obtained and prepared as described in Example 1.

Sample preparation:

ViaDerm-treated skin was washed with PBS and fixed for 4 hrs in 0.5% glutaraldehyde on ice. Fixed tissue was embedded in OCT medium (RA Lamb Limited, Eastbourne, UK) on Cardice and sectioned using a Leica CM3050S Cryostat.

ViaDerm parameters:

Parameter 3: ViaDerm parameter setting: 4 applications; Electrodes: 140; Burst length: 700μsec; Number of bursts: 3; TEWL control: 4.0 g/hm^2; TEWL after application(s): 30.3 g/hm^2.

Parameter 5: ViaDerm parameter setting: 4 applications; Electrodes: 140; Burst length: 700μsec; Number of bursts: 5; TEWL control: 6.7 g/hm^2; TEWL after application(s): 28.2 g/hm^2.

Parameter 6: ViaDerm parameter setting: 4 applications; Electrodes: 140; Burst length: 700μsec; Number of bursts: 1; TEWL control: 5.5 g/hm^2; TEWL after application(s): 24.8 g/hm^2.

Parameter 7: ViaDerm parameter setting: 4 applications; Electrodes: 140; Burst length: 700μsec; Number of bursts: 2; TEWL control: 5.2 g/hm^2; TEWL after application(s): 28.7 g/hm^2.

Parameter 10: ViaDerm parameter setting: 4 applications: Electrodes: 140; Burst length: 700μsec; Number of bursts: 5; TEWL control: 6.5 g/hm^2; TEWL after application(s): 32.6 g/hm^2.

Tissue staining:

a) Eosin: Tissue sections were collected onto adhesive-coated Histobond® microscope slides (RA Lamb), immersed in 1% aqueous eosin solution (BDH Laboratory Supplies, Dorset, UK) for 5 seconds, rinsed with water and allowed to dry.

b) Haematoxylin and Eosin (H&E): Tissue sections were collected onto Histobond® microscope slides, immersed in Harris' haematoxylin solution (BDH Laboratory Supplies) for 5 min, rinsed with water and allowed to dry. Post-staining with eosin was as above.

c) Toludine Blue: Tissue sections were collected onto Histobond® microscope slides, immersed in 1% aqueous toludine blue solution (TAAB Laboratories Equipment Limited, Berkshire, UK) for 5 min, rinsed with water and allowed to dry.

Results

FIG. 9 to FIG. 13 illustrate the dimensions of RF-micro channels that are created in human breast skin following application of ViaDerm at different parameter settings. The photomicrographs presented are representative of the entire population of channels

observed. In most cases the channels are approximately 100 μm in length and 30-50 μm at their widest aperture. The channels penetrate through the human epidermis and into the underlying dermis.

EXAMPLE 6

Co-localization of fluorescent nanoparticles into micro channels

The dimensions of the RF-micro channels were determined in order to evaluate whether they are of sufficient size to permit penetration and retention of 100 nm fluorescent nanoparticles.

Materials and Methods

Tissue:

Full-thickness human skin was obtained and prepared as described in Example 1.

ViaDerm parameters:

ViaDerm parameter setting: 4 applications; Electrodes: 140; Burst length: 700μsec; Number of bursts: 5; TEWL control: 6.7 g/hm^2; TEWL after application(s): 46.0 g/hm^2.

Sample preparation:

ViaDerm-treated skin was placed in a 6 well cell culture plate and maintained in 1.5 ml media (MEM (EAGLES) 25MM HEPES). 50 μl of a concentrated (4.57^{10}/μl) stock of fluorescently (FITC) labeled polystyrene nanospheres (L-1280; Sigma Chemical Company, Poole, UK) was pipetted onto the treated skin surface and the sample was incubated for 6 hrs at 37°C. After 6 hrs, additional 2 ml of media were added to submerge the skin. The skin was incubated for a further 18 hrs. Following two washes in PBS the skin was fixed in 0.5% gluataraldehyde for 1 hr on ice. Fixed tissue was embedded in OCT medium (RA Lamb) on Cardice and sectioned using a Leica CM3050S Cryostat.

Staining/visualization:

Sections were either stained with H&E and visualized as described in Example 5 or visualized unstained under blue fluorescence (Olympus BX50 microscope).

Results

FIG. 14 and FIG. 15 show that the RF-micro channels are of sufficient dimensions to uptake and entrap fluorescent nanoparticles with a diameter of 100 nm.

The micro channels are therefore of appropriate dimensions for the delivery of macromolecules and nanoparticles such as non-viral gene therapy vectors. Over the incubation period, many of the channels appear to have resealed at their surface to enclose the particles inside a 'drug delivery reservoir'.

EXAMPLE 7

Co-localization of fluorescently labeled DNA into micro channels

The aims of the following experiment were to determine whether the dimensions of the RF-micro channels are of sufficient dimensions to allow penetration and retention of fluorescently labeled plasmid DNA, and to determine whether complexation of plasmid DNA with lipid and polycation affects the penetration and retention of fluorescently labeled plasmid DNA.

Materials and Methods

Tissue:

Full-thickness human skin was obtained and prepared as described in Example 1.

ViaDerm parameters:

DNA alone: ViaDerm parameter setting: 2 applications; Electrodes: 140; Burst length: 700μsec; Number of bursts: 5; TEWL control: 7.5 g/hm^2; TEWL after application(s): 14.2 g/hm^2.

LPD: ViaDerm parameter setting: 4 applications; Electrodes: 140; Burst length: 700μsec; Number of bursts: 5; TEWL control: 5.3 g/hm^2; TEWL after application(s): 28.3 g/hm^2.

Sample preparation:

Fluorescently labeled plasmid DNA was prepared by labeling pEGFP-N1 reporter plasmid with a rhodamine fluorophore using a LabelIT® Nucleic Acid Labeling Kit (Mirus Corporation, Madison, WI). ViaDerm-treated skin was placed in a 6 well cell culture plate and maintained in 1.5 ml media (MEM (EAGLES) 25MM HEPES). 50μl of labeled pDNA alone (containing 2μg pDNA) or a suspension of LPD complexes comprising fluorescently labeled pDNA (5μg) pre-complexed with protamine sulphate (10μg; Grade X from salmon sperm; Sigma Chemicals, Poole, UK) and 1,2-Dioleoyl-3-Trimethylammonium-Propane (DOTAP) liposomes (15μg; Avanti Polar Lipids, Alabama, USA) were pipetted onto the treated skin surface and the sample was

incubated for 1 hr at 37°C. After 1 hr, additional 2 ml of media were added to submerge the skin. The skin was incubated for a further 47 hrs. Following two washes in PBS the skin was fixed in 0.5% glutaraldehyde for 1 hr on ice. Fixed tissue was embedded in OCT medium (RA Lamb) on Cardice and sectioned using a Leica CM3050S Cryostat.

Light/fluorescence microscopy:

Sections were visualized unstained under brightfield or blue fluorescence (Olympus BX50 microscope).

Results

FIG. **16A** shows the distribution of fluorescently labeled pDNA on a flat section of skin. The skin autofluoresces green whilst the rhodamine-labeled pDNA is red. Where overlap between the two fluorescent species occurs the underlying region appears as yellow. FIG. **16B** shows a fluorescent pDNA particle residing in a skin fold (note the stratum corneum is continuous and unbroken). FIG. **16C** shows an RF-micro channel. Interestingly, FIG. **16C** shows the presence of a fluorescently labeled pDNA particle deep in the dermis of the same ViaDerm-treated tissue sample shown in FIG. **16C**.

FIG. **17** shows that the fluorescence associated with the rhodamine labeled pDNA is not diminished by complexation with polycation and lipid into an LPD gene delivery vector. In these images the LPD vector is clearly seen to be localized both at the surface (FIG. **17B** and **17D**) and inside (FIG. **16F**) the RF-micro channels.

EXAMPLE 8

β-galactosidase gene expression in heat-separated epidermal membrane

It was next determined whether β-galactosidase reporter gene can be expressed in heat-separated epidermal membrane when lipid:polycation:pDNA (LPD) vectors are applied to skin pre-treated with ViaDerm.

Materials and Methods

Tissue:

Full-thickness human skin was obtained and prepared as described in Example 2.

ViaDerm parameters:

ViaDerm parameter setting: 2 applications; Electrodes: 140; Burst length: 700μsec; Number of bursts: 5; TEWL control: 6.0 g/hm^2; TEWL after application(s): 14.6 g/hm^2.

Sample preparation:

ViaDerm-treated skin was placed in a 6 well cell culture plate and maintained in 1.5 ml media (MEM (EAGLES) 25MM HEPES). pCMVβ plasmid DNA, expressing the β-galactosidase reporter gene under the control of the human cytomegalovirus immediate early (CMV IE) promoter, was obtained from ClonTech Laboratories Inc. (Palo Alto, USA), propagated using a transformed DH5α strain of *Eschericia coli* colonized onto an ampicillin selective LB agar plate and cultured overnight at 37°C. The pDNA was harvested and purified using a Qiagen Plasmid Mega Kit (Qiagen, Crawley, UK).

A 300 µl suspension of lipid:polycation:pDNA (LPD) vectors was prepared containing 50 µg pCMVβ plasmid DNA, 100 µg protamine sulphate (Grade X from salmon sperm) and 150 µg DOTAP liposomes. LPD complexes were prepared by sequential addition (with 10 min incubation at each step) of firstly protamine (1 mg/ml stock in sterile purified water) and then extruded DOTAP liposomes to pDNA (1 mg/ml stock in TE buffer) to achieve a lipid:protamine:pDNA mass (w/w) ratio of 3:2:1. 100 µl of the LPD suspension was pipetted onto the ViaDerm-treated skin surface and the sample was incubated for 18 hrs at 37°C. At 18 hrs, additional 2 ml of media were added and the submerged skin was incubated for a further 30 hrs.

X-gal staining:

Following two washes in PBS the skin was fixed on ice in 0.5% glutaraldehyde for 1 hr and stained for β-galactosidase activity over 24 hrs using a LacZ Reporter Assay Kit for Tissue Staining (Invivogen, San Diego, CA). Following two further washes in PBS the skin was heat-separated as described in Example 2.

Light microscopy:

The heat-separated epidermal membrane was visualized using an Olympus BX50 microscope.

Results

FIG. **18** shows the appearance of micro channels in heat-separated epidermal membrane. The brown discoloration observed previously in Example 4 is once again apparent. The boundary, and possibly the center, of the channel are colored blue. Under the enzymatic assay conditions blue coloration is associated with the presence of the reporter gene product, β-galactosidase.

EXAMPLE 9

β-galactosidase gene expression en face

It was then determined whether β-galactosidase reporter gene can be delivered to, and expressed in, full-thickness skin following application of the ViaDerm device.

Materials and Methods

Tissue:

Full-thickness human skin was obtained and prepared as described in Example 2.

ViaDerm parameters:

ViaDerm parameter setting: 2 applications; Electrodes: 140; Burst length: 700μsec; Number of bursts: 5; TEWL control: 10.3 g/hm^2; TEWL after application(s): 26.2 g/hm^2.

Sample preparation:

ViaDerm-treated skin was placed in a 6 well cell culture plate and maintained in 1.5 ml media (MEM (EAGLES) 25MM HEPES). 100 μl of pCMVβ plasmid DNA solution (1 mg/ml) was pipetted onto the ViaDerm-treated skin surface and the sample was incubated for 24 hrs at 37°C. Following two washes in PBS the stratum corneum of the skin was removed by tape stripping 20 times with D-Squame adhesive tape (CuDerm Corp., Dallas, TX).

X-gal staining:

The tissue was fixed on ice in 0.5% glutaraldehyde for 1 hr, rinsed overnight in PBS and stained for β-galactosidase expression over 48 hrs using a LacZ Reporter Assay Kit for Tissue Staining, (Invivogen, San Diego, CA).

Light microscopy:

The surface of the tape-stripped skin was visualized using an Olympus BX50 microscope and an Scholt KL1500 electronic light source.

Results

FIG. 19 shows the presence of expressed β-galactosidase in the RF-micro channels. The skin was tape-stripped following application of the ViaDerm device and the DNA solution to allow more stain to reach the appropriate area of the tissue. In this experiment the plasmid was used alone, i.e. without any non-viral carrier system, as numerous studies have shown the ability of naked DNA to be expressed efficiently in *vivo* (e.g. Hengge, U.R. et al. Nature Genet. 10: 161, 1995; Hengge, U.R. et al. J.

Clinical Investigation, 97: 2911, 1996; and Chesnoy, S. and Huang, L. Molecular Therapy, 5: 57, 2002).

EXAMPLE 10

β-galactosidase gene expression in sectioned tissue

The pattern of β-galactosidase reporter gene expression in ViaDerm-treated skin was detected.

Materials and Methods

Tissue:

Full-thickness human skin was obtained and prepared as described in Example:

ViaDerm parameters:

H&E stained: ViaDerm parameter setting: 2 applications; Electrodes: 140; Burst length: 700μsec; Number of bursts: 5; TEWL control: 11.0 g/hm^2; TEWL after application(s): 20.6 g/hm^2.

Unstained: ViaDerm parameter setting: 2 applications; Electrodes: 140; Burst length: 700μsec; Number of bursts: 5; TEWL control: 8.3 g/hm^2; TEWL after application(s): 26.9 g/hm^2.

Sample preparation:

ViaDerm-treated skin was placed in a 6 well cell culture plate and maintained in 1.5 ml media (MEM (EAGLES) 25MM HEPES). 100 μl of pCMVβ plasmid DNA solution (1mg/ml) was pipetted onto the ViaDerm-treated skin surface and the sample was incubated for 24 hrs at 37°C. Following two washes in PBS the tissue was fixed on ice in 0.5% glutaraldehyde for 1 hr, rinsed overnight in PBS and stained for β-galactosidase expression over 24 hrs using a LacZ Reporter Assay Kit for Tissue Staining, (Invivogen, San Diego, CA).

Post-staining:

Tissue sections were post-stained with H&E, as appropriate, as described in Example 5.

Light microscopy:

The tissue sections were visualized using an Olympus BX50 microscope.

Results

FIG. **20** shows the presence of blue staining, relating to reporter gene expression, in the cells surrounding the RF-micro channel. The staining is restricted to the dermis and does not appear to disperse into the surrounding tissue. FIG. **20A** shows the appearance of an RF-micro channel without post-staining with H&E. In this case the reporter gene expression appears to be in the cells of the epidermis in the resealing tissue directly above the micro channel. No dermal staining is observed. FIG. **20B** acts as an internal control demonstrating that no reporter gene associated blue staining is apparent in skin folds in close proximity to the micro channel.

It will be appreciated by persons skilled in the art that the present invention is not limited by what has been particularly shown and described herein above. Rather the scope of the invention is defined by the claims that follow.

CLAIMS

1. A transdermal delivery system for an oligonucleotide or polynucleotide comprising: an apparatus for facilitating transdermal delivery of an oligonucleotide or polynucleotide through skin of a subject, the apparatus capable of generating at least one micro-channel in an area on the skin of the subject, and a pharmaceutical composition comprising at least one oligonucleotide or polynucleotide.

2. The system according to claim 1 wherein the oligonucleotide or polynucleotide is selected from oligonucleotides or polynucleotides of DNA and RNA, and synthetic analogs comprising non-natural linkages.

3. The system according to claim 2 wherein the oligonucleotide or polynucleotide encodes at least one polypeptide or a fragment thereof.

4. The system according to claim 2 wherein the oligonucleotide or polynucleotide is operably linked to regulatory sequences thereby capable of being expressed in cells of the subject.

5. The system according to claim 1 wherein the oligonucleotide or polynucleotide is selected from oligonucleotides or polynucleotides encoding insulin, proinsulin, follicle stimulating hormone, insulin like growth factor-1, insulin like growth factor-2, platelet derived growth factor, epidermal growth factor, fibroblast growth factors, nerve growth factor, colony stimulating factors, transforming growth factors, tumor necrosis factor, calcitonin, parathyroid hormone, growth hormone, bone morphogenic protein, erythropoietin, hemopoietic growth factors, luteinizing hormone, calcitonin; glucagon; clotting factors, anti-clotting factors, atrial natriuretic factor, plasminogen activators, bombesin, thrombin, enkephalinase, vascular endothelial growth factor, interleukins, viral antigens, non-viral antigens, transport proteins, antibodies, and fragments thereof.

6. The system according to claim 2 wherein the oligonucleotide is an antisense oligonucleotide.

7. The system according to any one of claims 1 to 6 wherein the pharmaceutical composition further comprises at least one additive selected from lipids, polycations, and nuclease inhibitors.

8. The system according to claim 1 wherein the apparatus comprises:
   a. an electrode cartridge comprising at least one electrode; and
   b. a main unit comprising a control unit which is adapted to apply electrical energy to the electrode when the electrode is in vicinity of the skin, typically generating current flow or one or more sparks, enabling ablation of stratum corneum in an area beneath the electrode, thereby generating at least one micro-channel.

9. The system according to claim 8 wherein the electrode cartridge comprises a plurality of electrodes capable of generating a plurality of micro-channels of uniform shape and dimensions.

10. The system according to claim 9 wherein the electrical energy is of radio frequency.

11. A method for transdermal delivery of an oligonucleotide or polynucleotide comprising:
   (a) generating at least one micro-channel in an area of the skin of a subject; and
   (b) applying a pharmaceutical composition comprising a therapeutically effective amount of an oligonucleotide or polynucleotide.

12. The method according to claim 11 wherein the oligonucleotide or polynucleotide is selected from oligonucleotides or polynucleotides of DNA and RNA, and a synthetic analog thereof comprising non-natural linkages.

13. The method according to claim 12 wherein the oligonucleotide or polynucleotide encodes at least one polypeptide or a fragment thereof.

14. The method according to claim 12 wherein the oligonucleotide or polynucleotide is operably linked to regulatory sequences thereby capable of being expressed in cells of the subject.

15. The method according to claim 12 wherein the oligonucleotide or polynucleotide is selected from oligonucleotides or polynucleotides encoding insulin, proinsulin, follicle stimulating hormone, insulin like growth factor-1, insulin like growth factor-2, platelet derived growth factor, epidermal growth factor, fibroblast growth factors, nerve growth factor, colony stimulating factors, transforming growth factors, tumor necrosis factor, calcitonin, parathyroid hormone, growth hormone, bone morphogenic protein, erythropoietin, hemopoietic growth factors, luteinizing hormone, calcitonin; glucagon; clotting factors, anti-clotting factors, atrial natriuretic factor, plasminogen activators, bombesin, thrombin, enkephalinase, vascular endothelial growth factor, interleukins, viral antigens, non-viral antigens, transport proteins, antibodies, and fragments thereof.

16. The method according to claim 12 wherein the oligonucleotide is an antisense oligonucleotide.

17. The method according to any one of claims 11 to 16 wherein the micro channels are generated using radio frequency energy.

18. The method according to any one of claims 11 to 17, substantially as shown in the specification.

For the applicants:

Cynthia Webb
Webb & Associates
Patent Attorneys

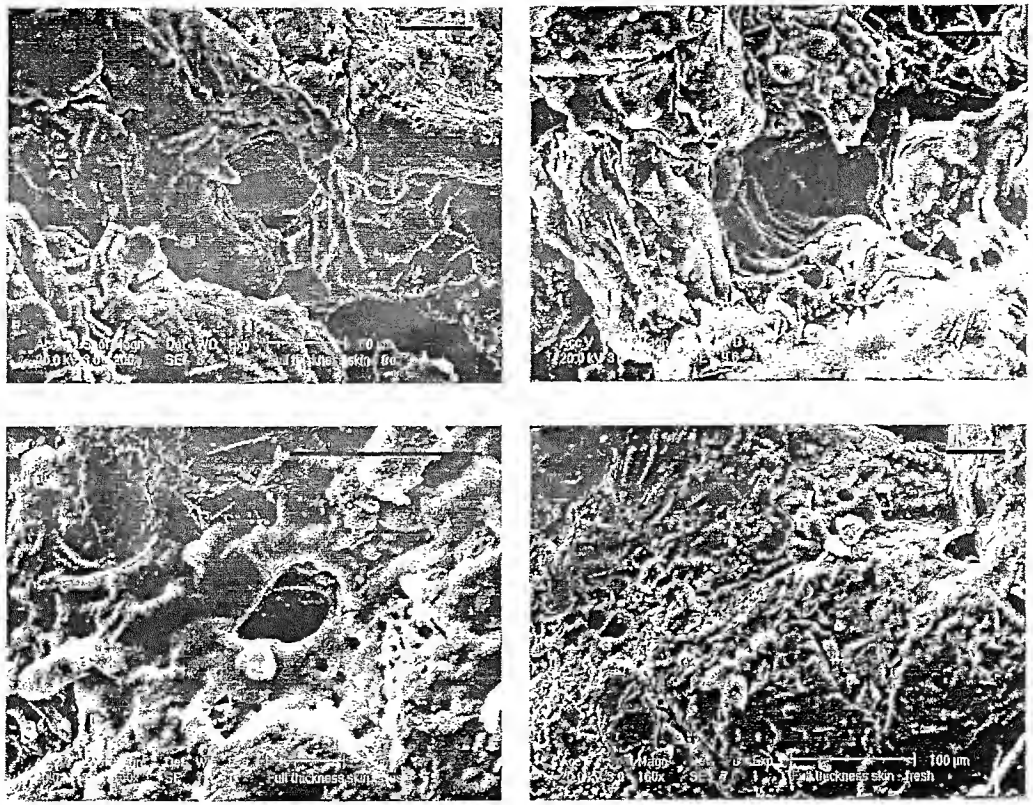


FIG. 1

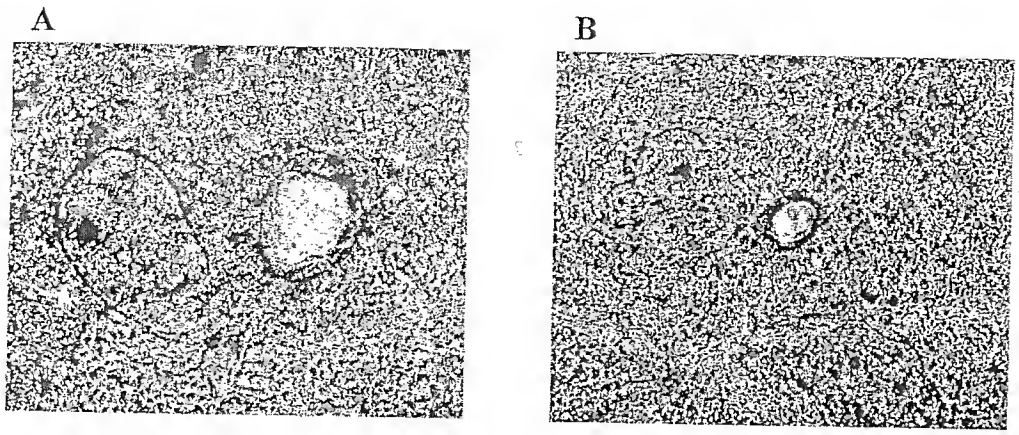


FIG. 2

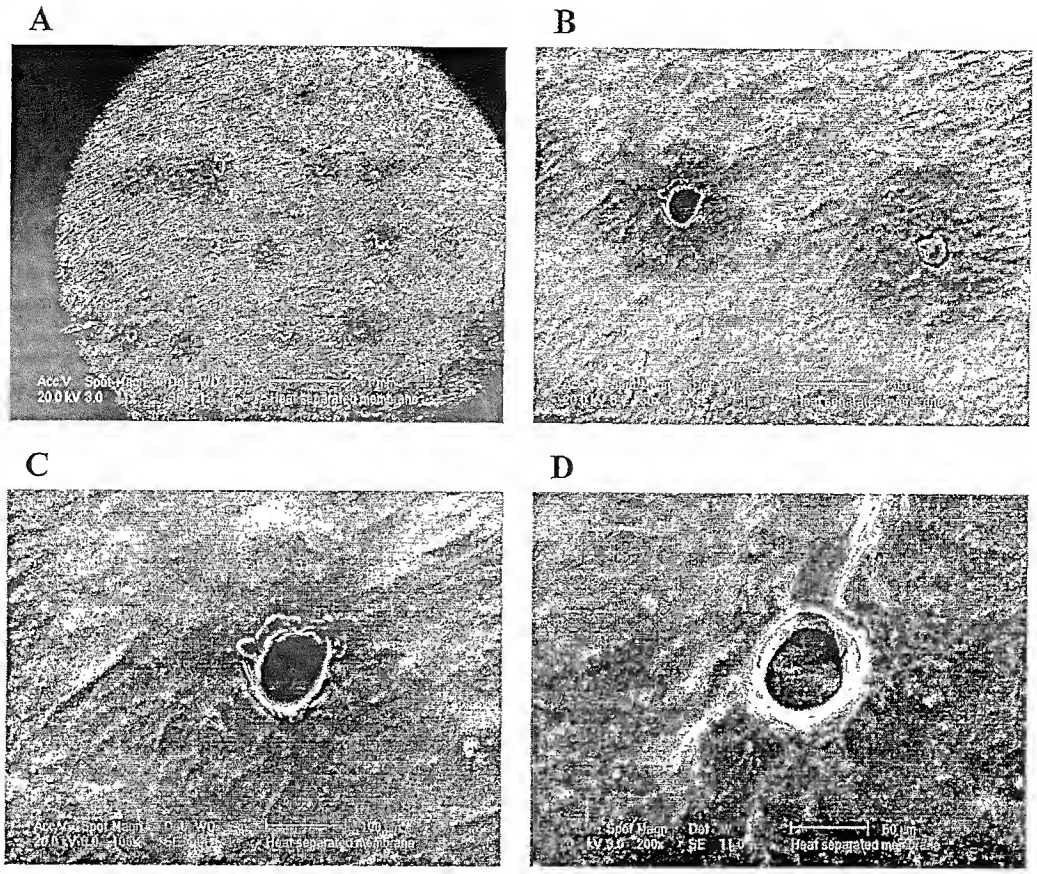
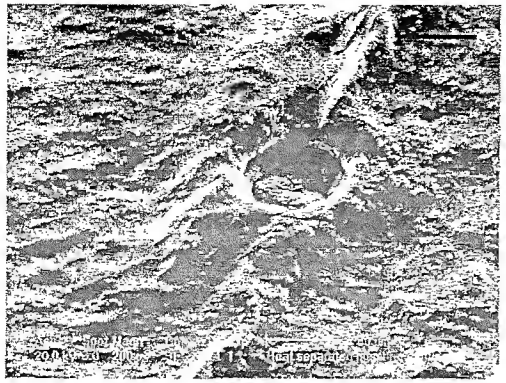


FIG. 3A-D

E



F

FIG. 3E-F

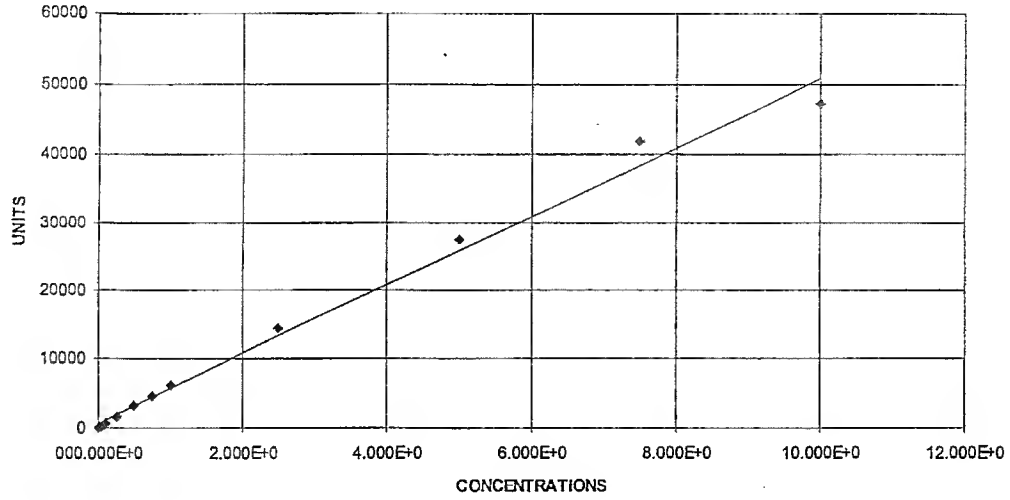

60000
50000
40000
30000
20000
10000
0
UNITS
000.000E+0
2.000E+0
4.000E+0
6.000E+0
8.000E+0
10.000E+0
12.000E+0
CONCENTRATIONS

FIG. 4

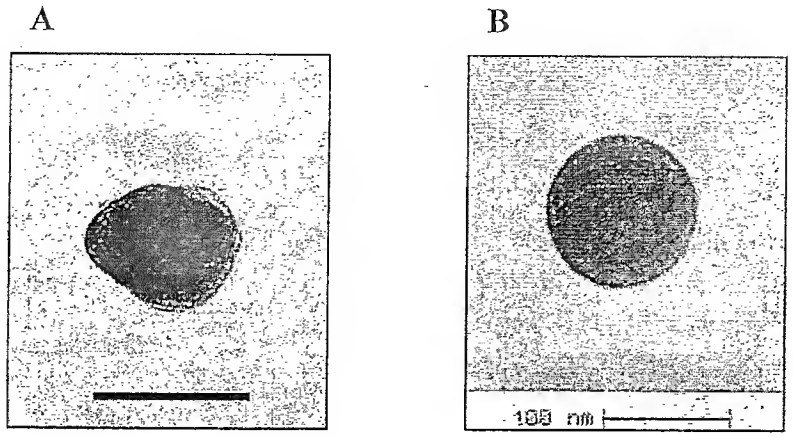


FIG. 5

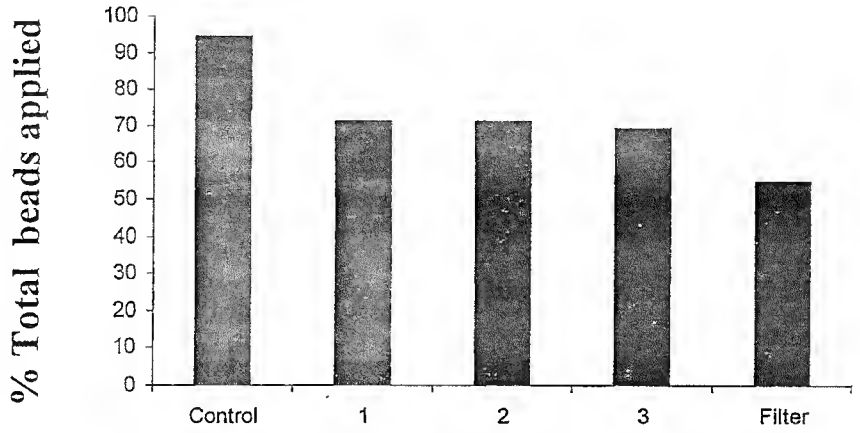

% Total beads applied
100
90
80
70
60
50
40
30
20
10
0
Control
1
2
3
Filter

FIG. 6

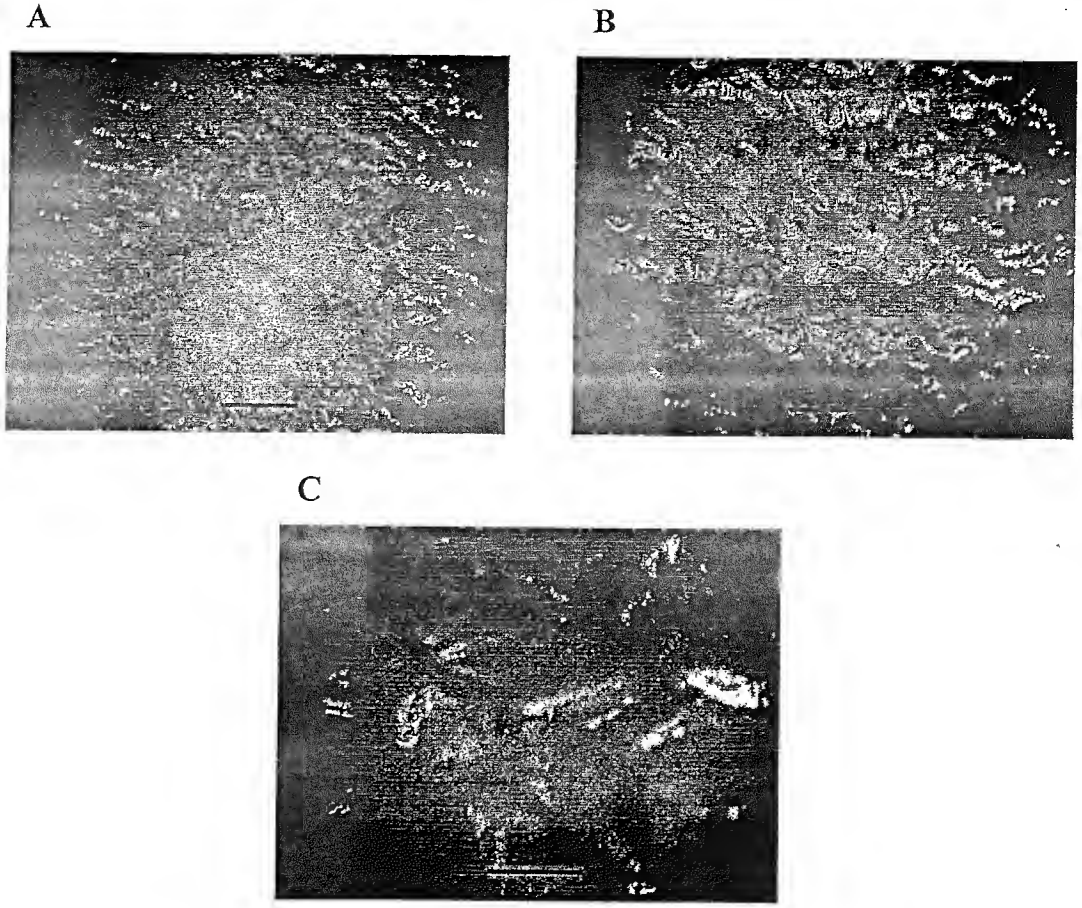


FIG. 7

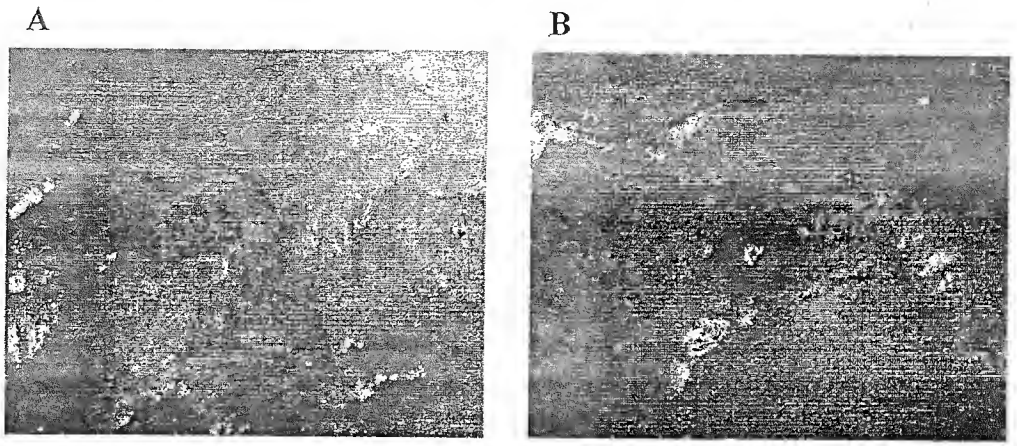


FIG. 8

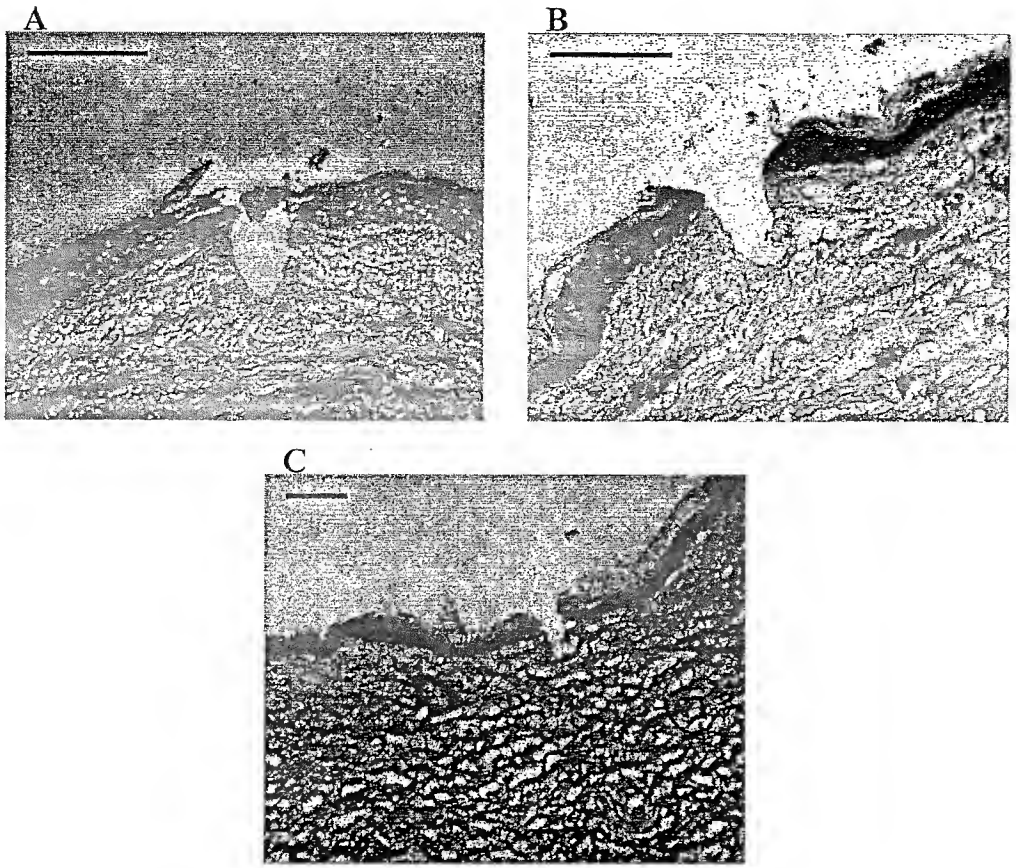


FIG. 9

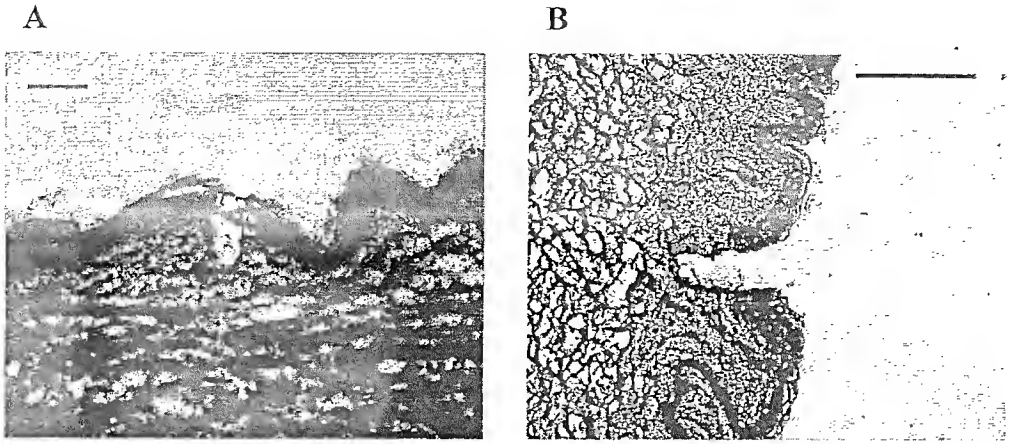


FIG. 10

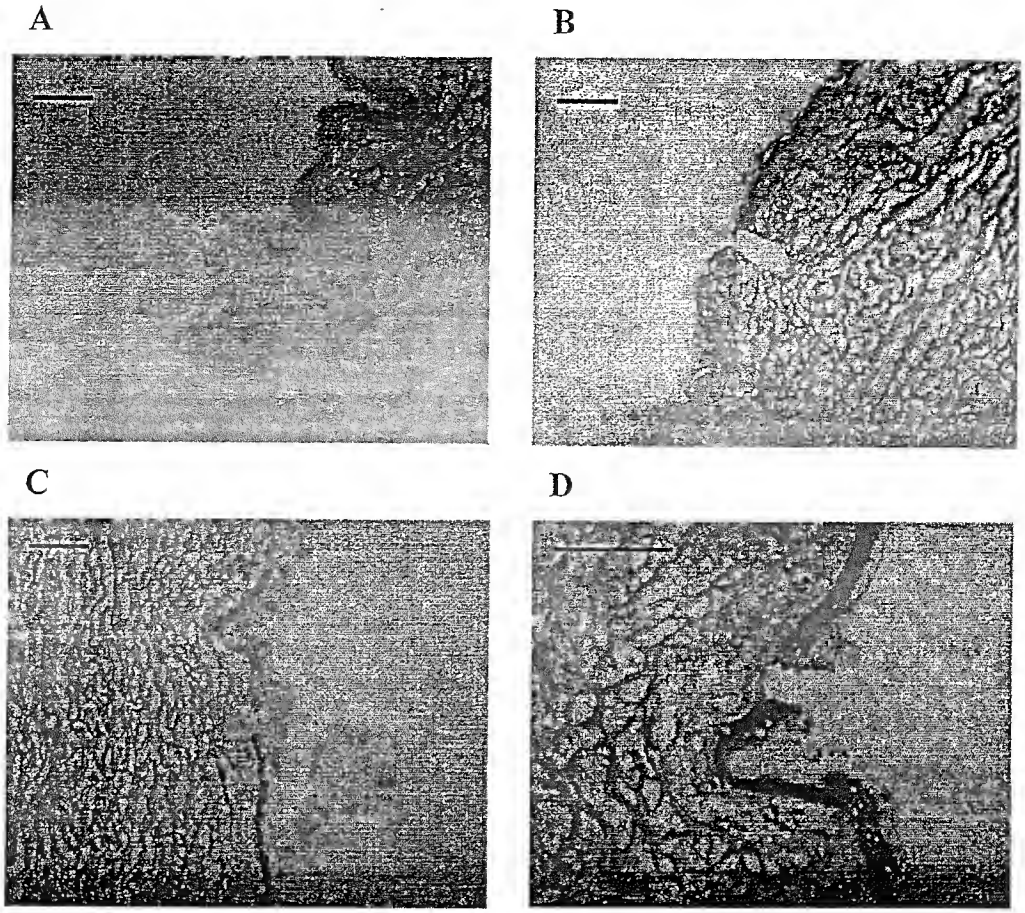


FIG. 11

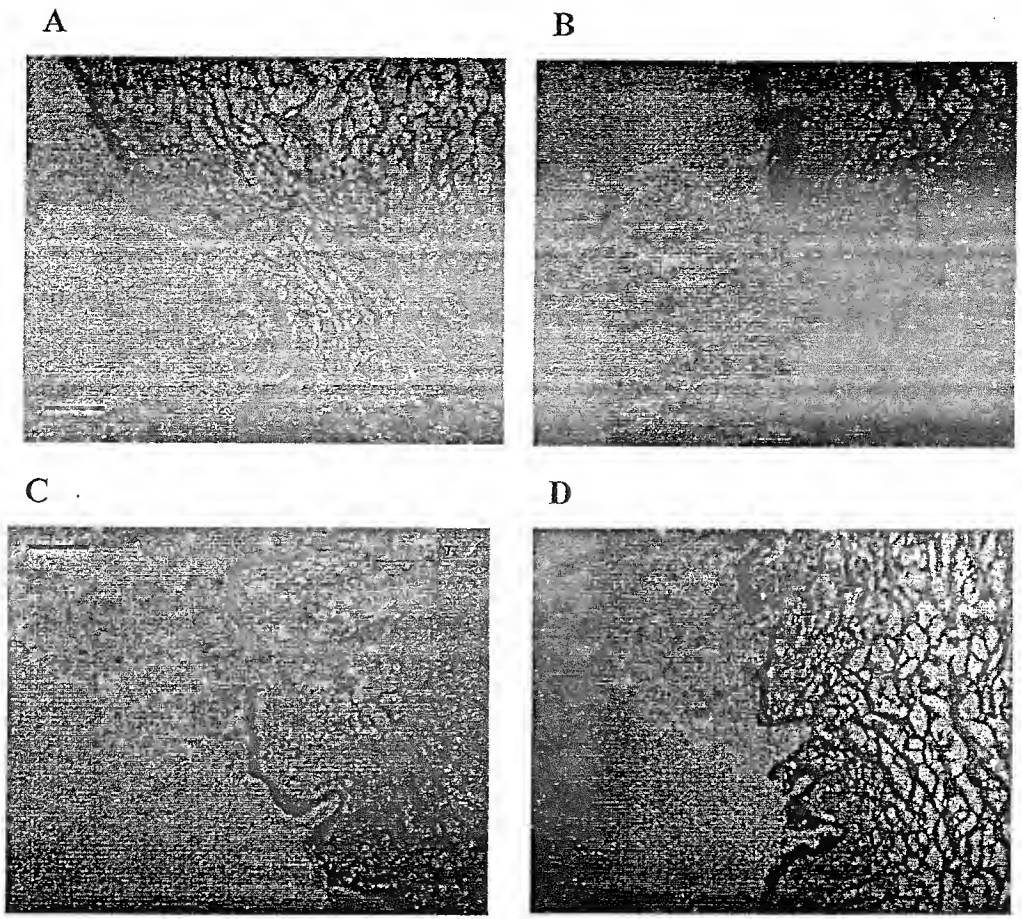


FIG. 12

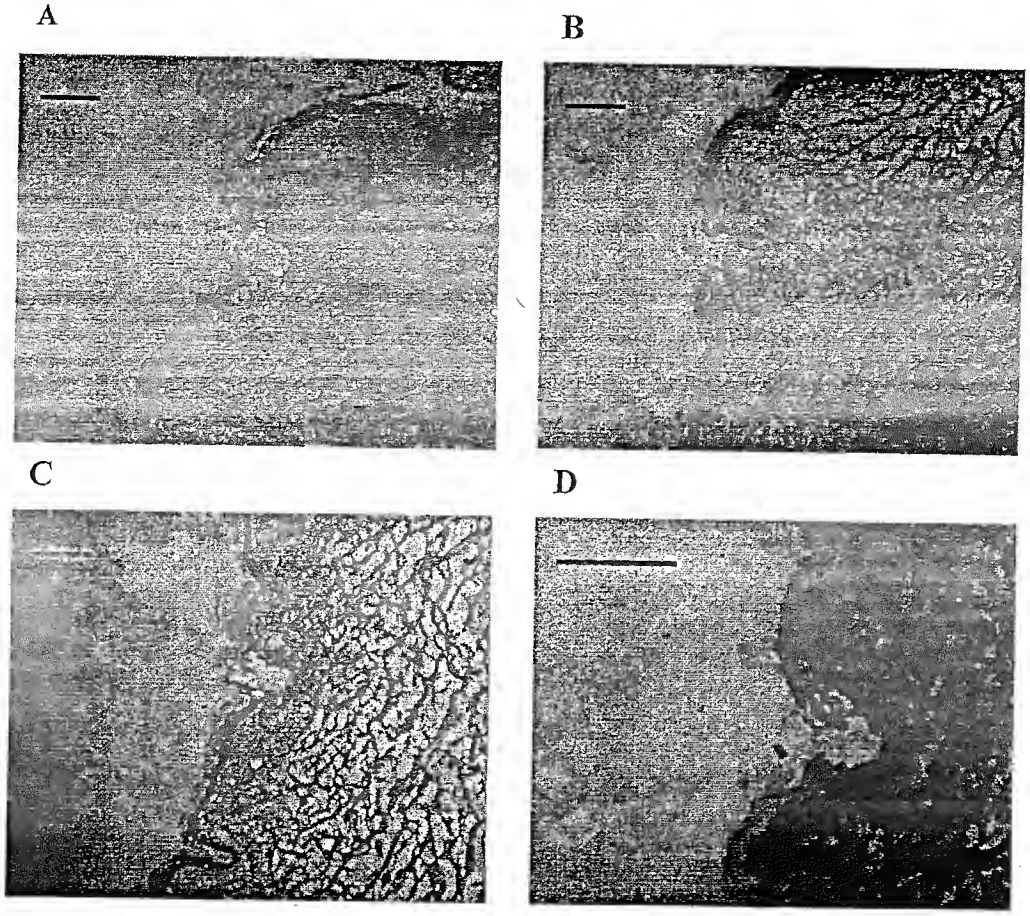


FIG. 13

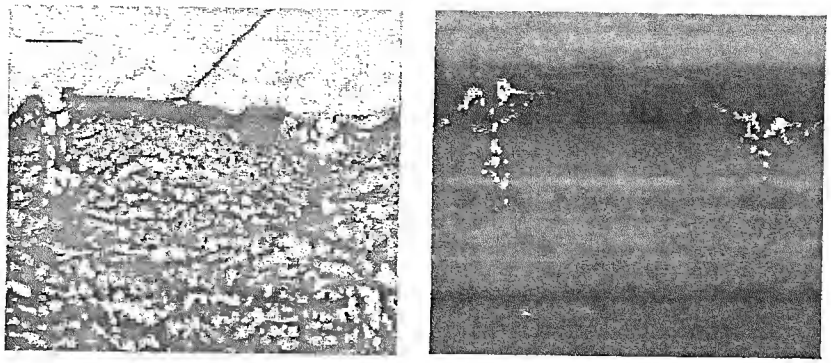


FIG. 14

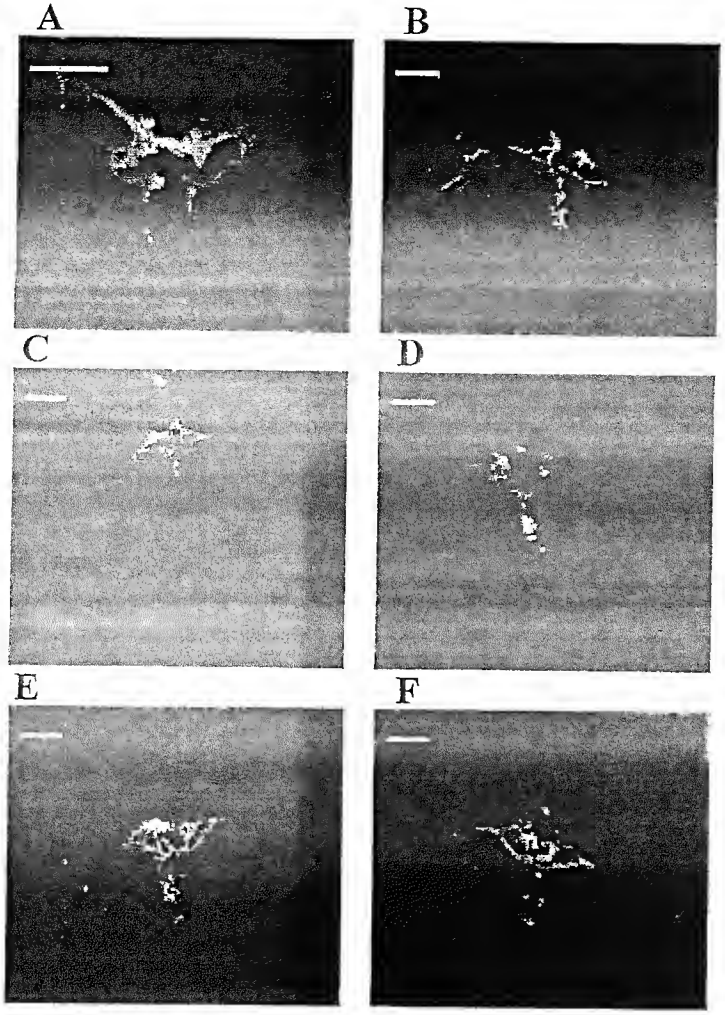


FIG. 15

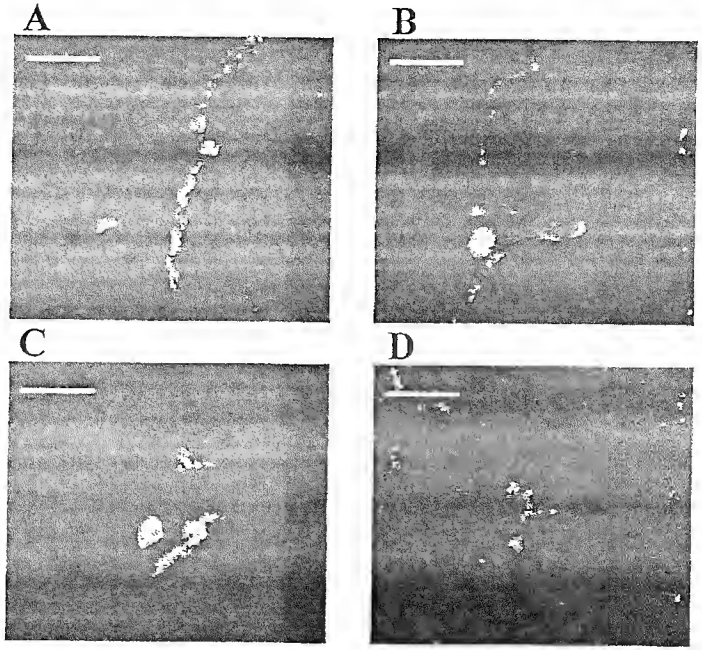


FIG. 16

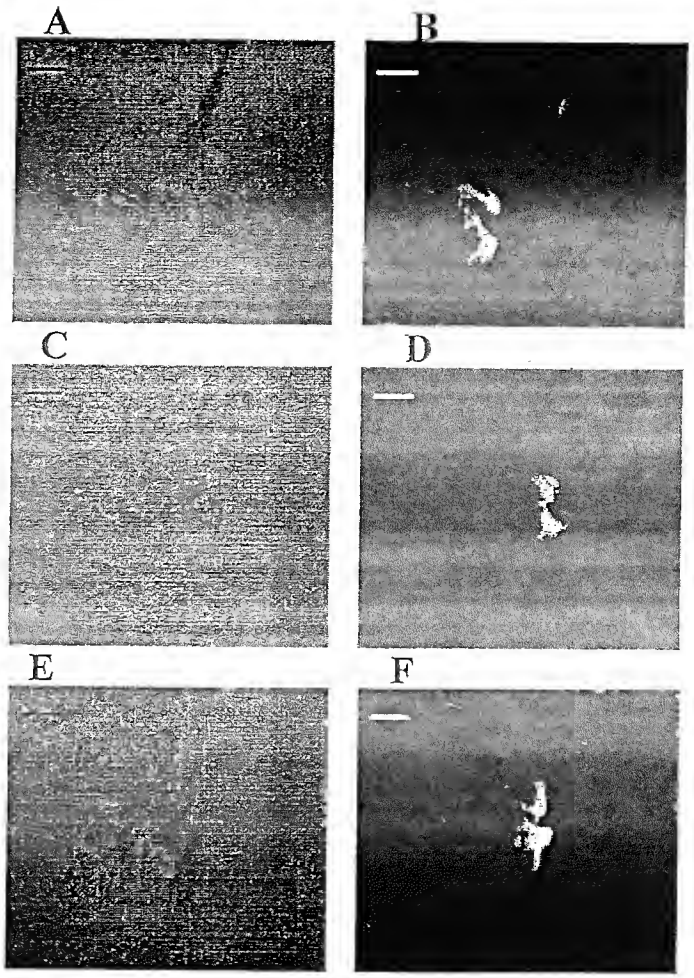


FIG. 17

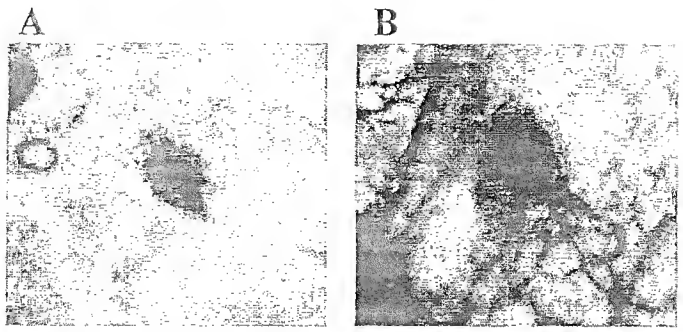


FIG. 18

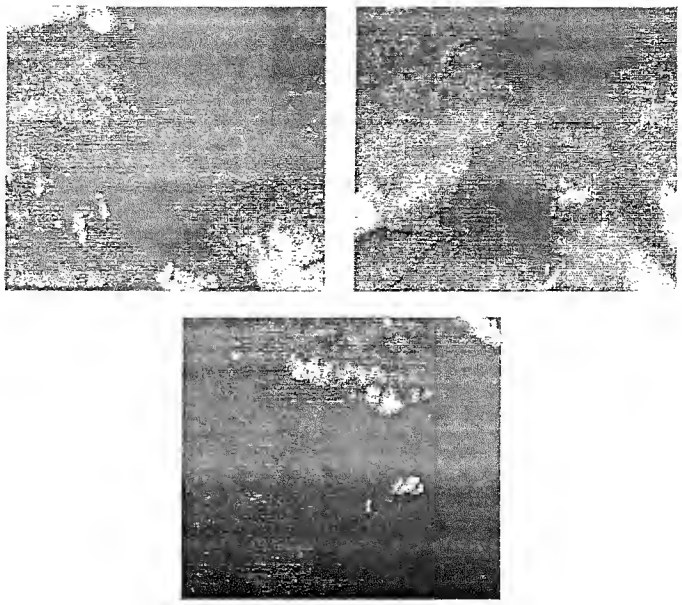


FIG. 19

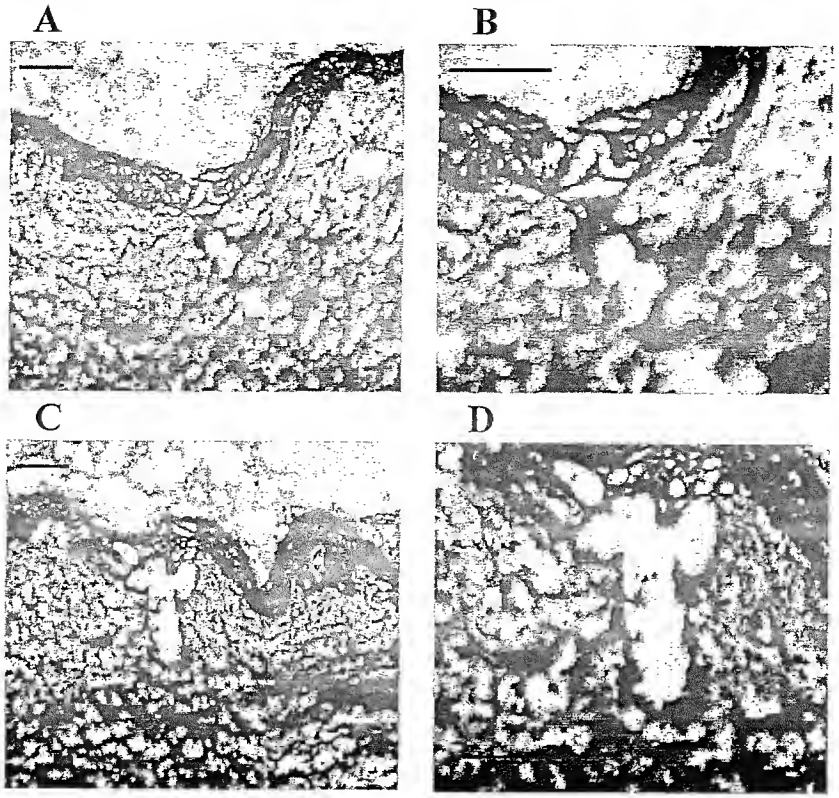


FIG. 20

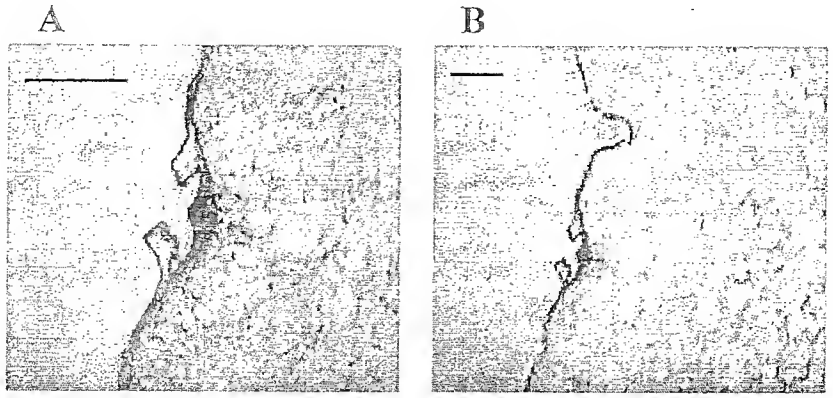


FIG. 21